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(12) **United States Patent**  
**Stadheim et al.**(10) **Patent No.:** **US 9,187,552 B2**  
(45) **Date of Patent:** **Nov. 17, 2015**(54) **METHOD FOR PREPARING ANTIBODIES  
HAVING IMPROVED PROPERTIES**(75) Inventors: **Terrance A. Stadheim**, Lyme, NH (US);  
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Rahway, NJ (US)(\*) Notice: Subject to any disclaimer, the term of this  
patent is extended or adjusted under 35  
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(21) Appl. No.: **13/699,213**(22) PCT Filed: **May 25, 2011**(86) PCT No.: **PCT/US2011/037826**§ 371 (c)(1),  
(2), (4) Date: **Nov. 20, 2012**(87) PCT Pub. No.: **WO2011/149999**PCT Pub. Date: **Dec. 1, 2011**(65) **Prior Publication Data**

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27, 2010.(51) **Int. Cl.****C07K 1/00** (2006.01)  
**C07K 16/00** (2006.01)  
**C12P 21/08** (2006.01)  
**C07K 16/24** (2006.01)  
**C07K 16/32** (2006.01)  
**A61K 39/00** (2006.01)(52) **U.S. Cl.**CPC ..... **C07K 16/00** (2013.01); **C07K 16/241**  
(2013.01); **C07K 16/32** (2013.01); **A61K**  
**2039/505** (2013.01); **C07K 2317/41** (2013.01);  
**C07K 2317/71** (2013.01)(58) **Field of Classification Search**None  
See application file for complete search history.(56) **References Cited**

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*Primary Examiner* — Chun Dahle(74) *Attorney, Agent, or Firm* — Gloria Fuentes; Immac  
Thampoe(57) **ABSTRACT**The present invention is directed to methods and composi-  
tions for the production of Fc-containing polypeptides having  
improved properties and comprising mutations at positions  
243 and 264 of the Fc region.**8 Claims, 34 Drawing Sheets**

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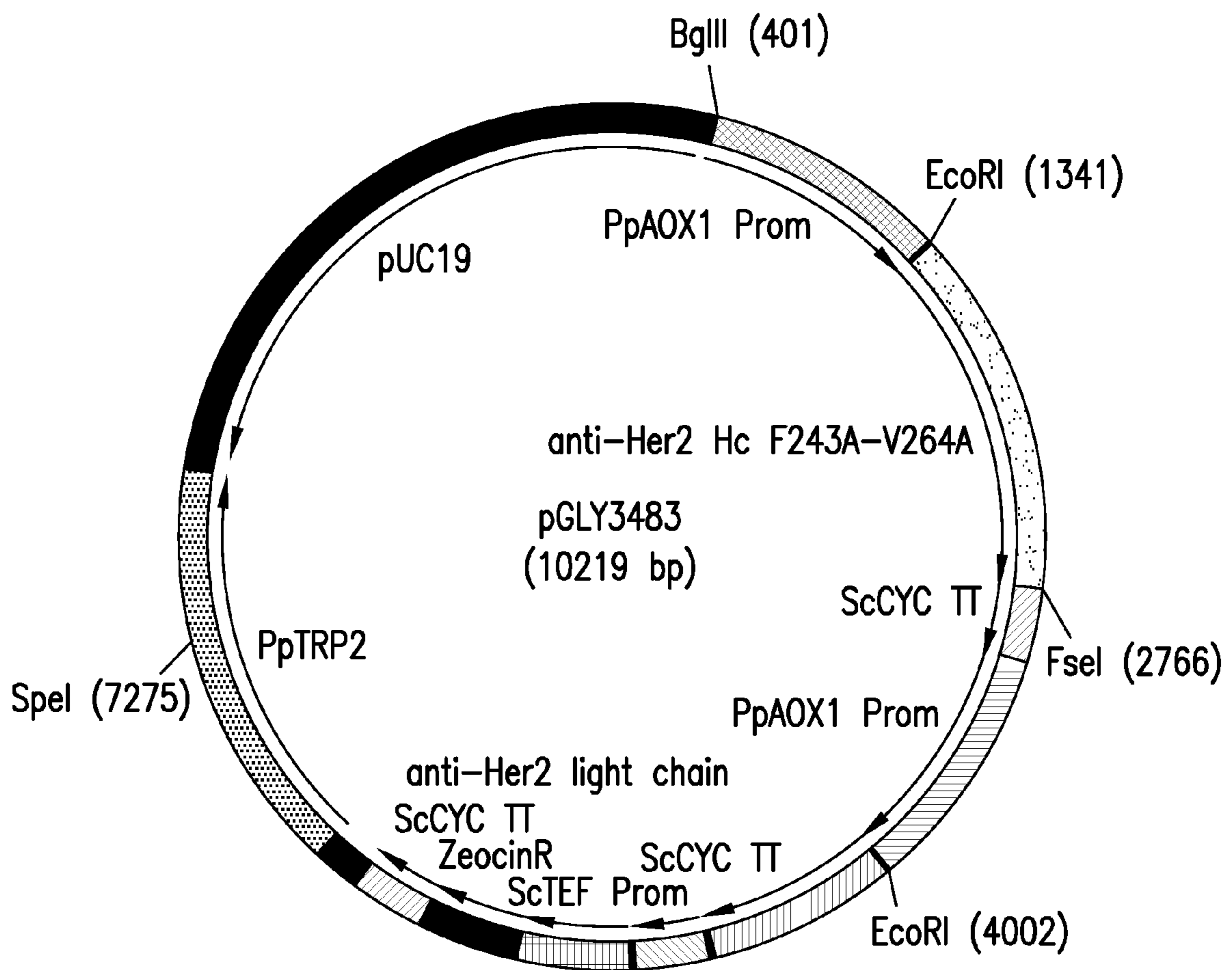


FIG. 1

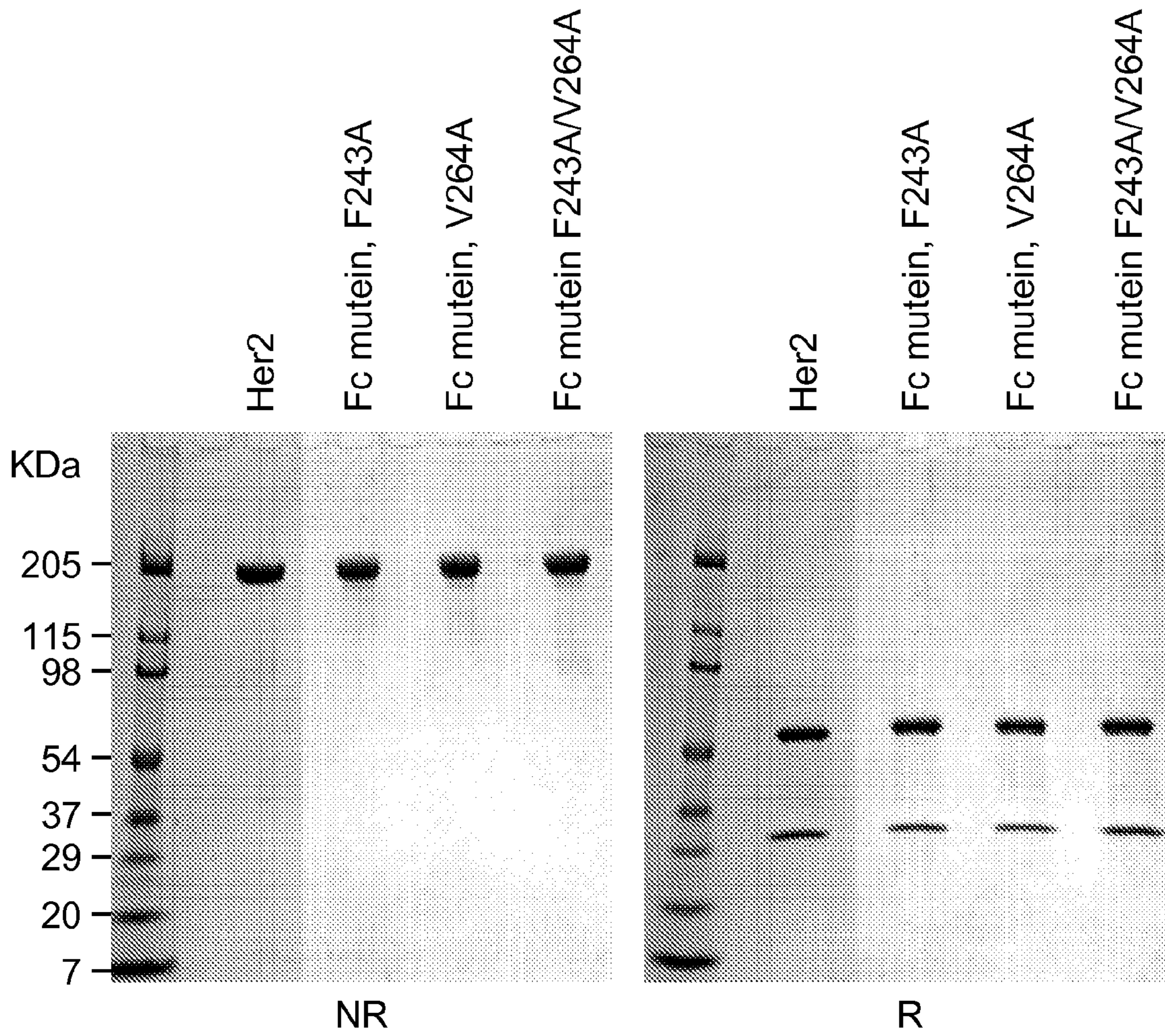


FIG.2

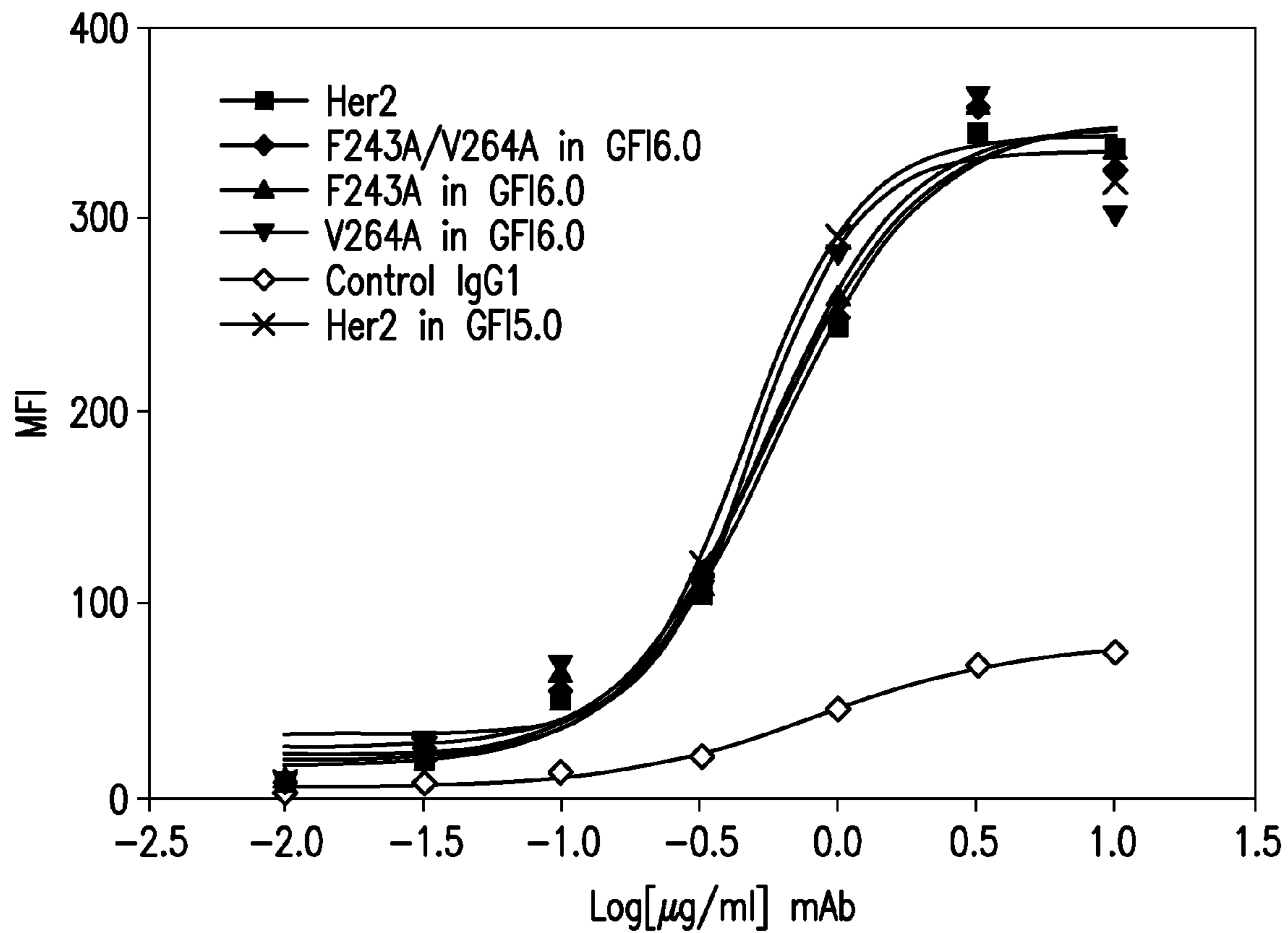


FIG.3

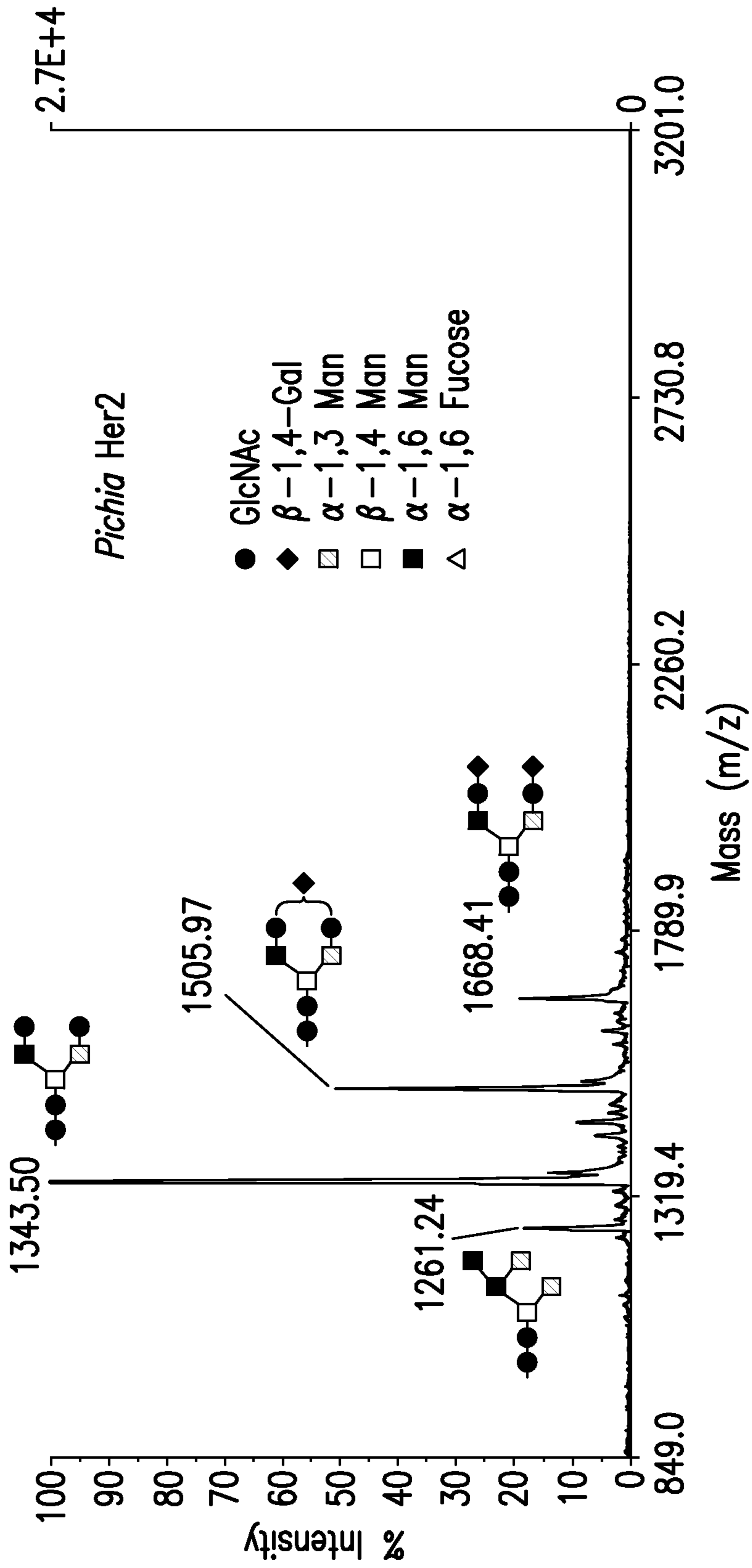


FIG. 4

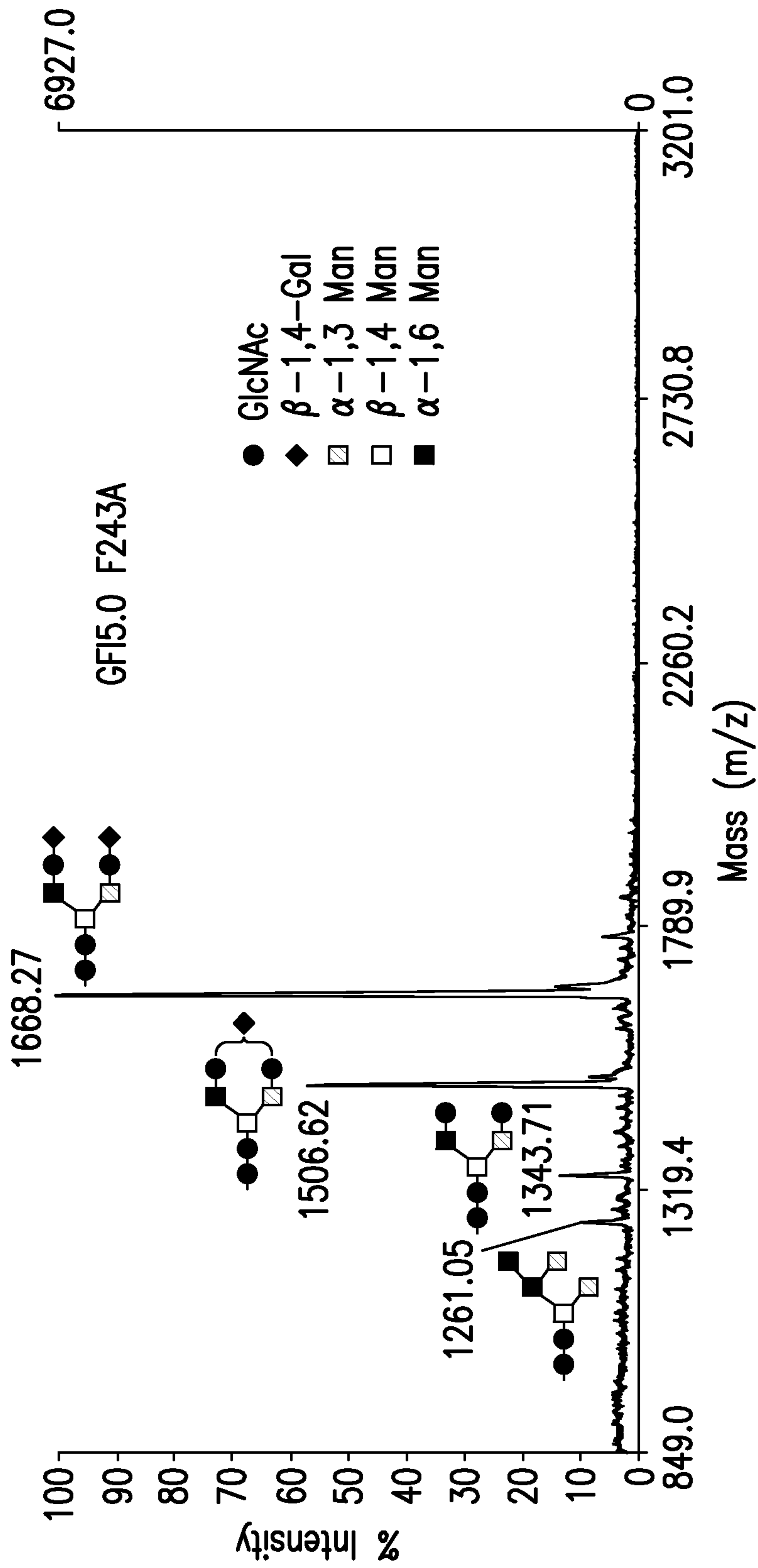


FIG. 5

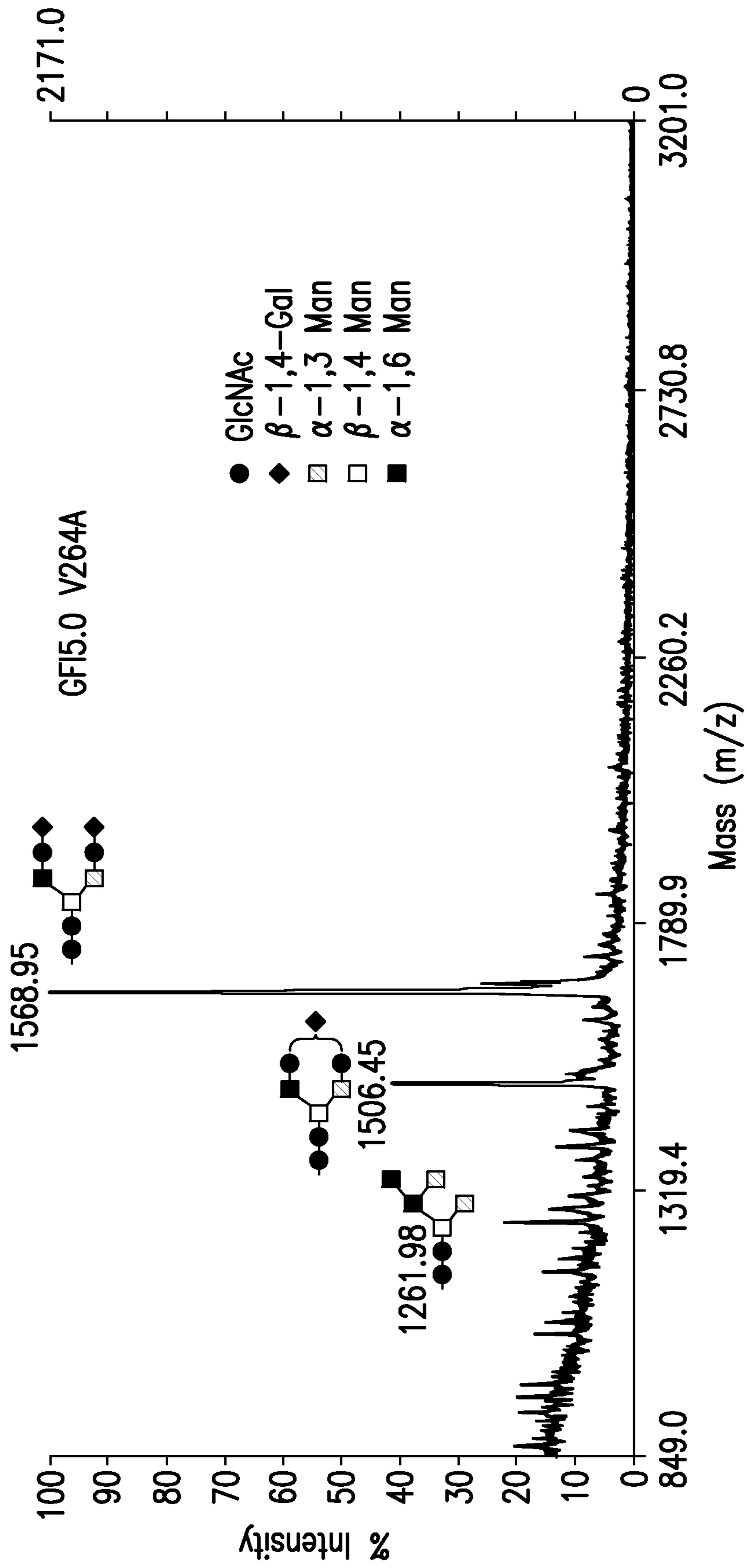


FIG. 6



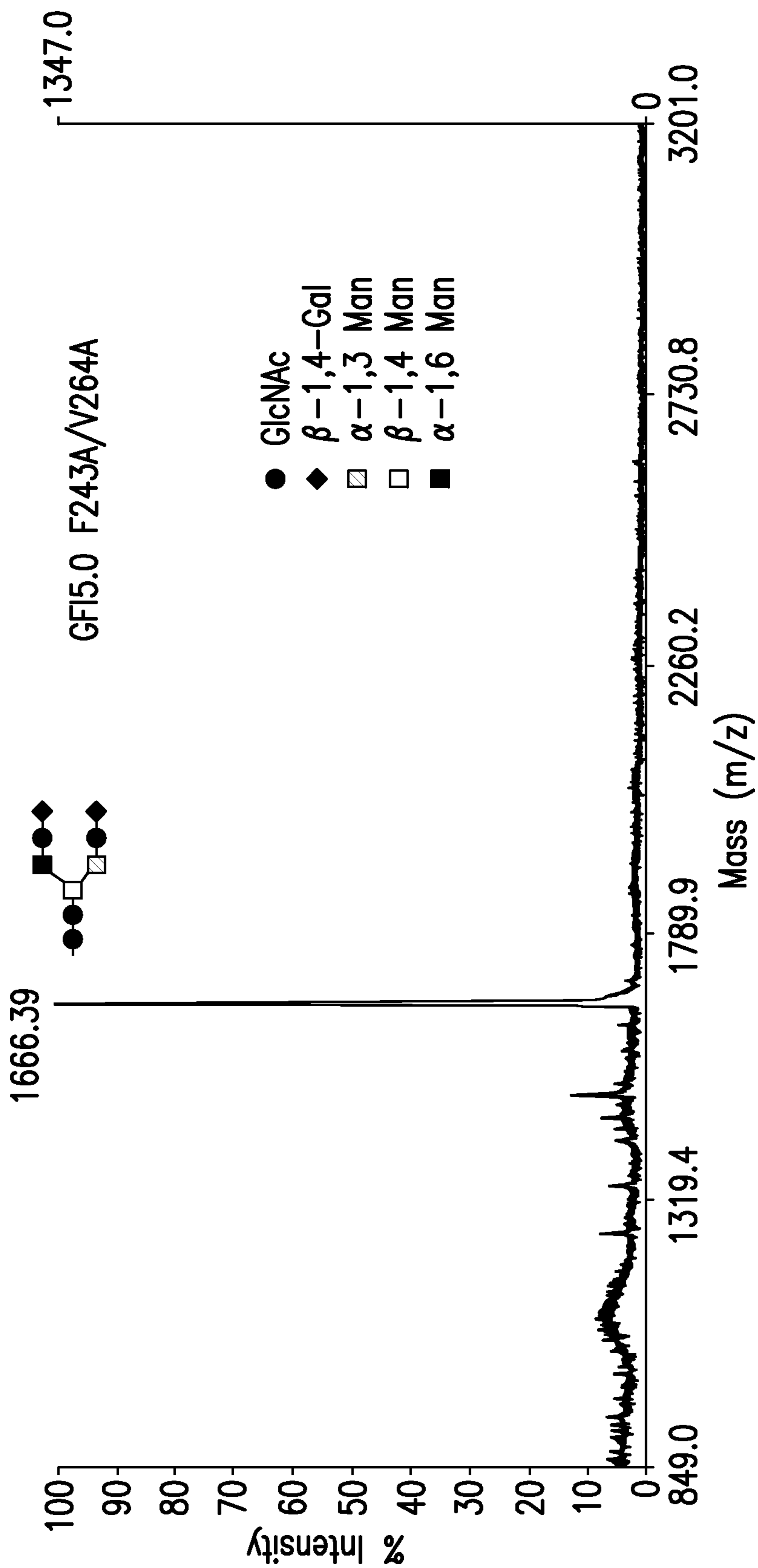


FIG. 7

GFI 6.0 F243A/V264A

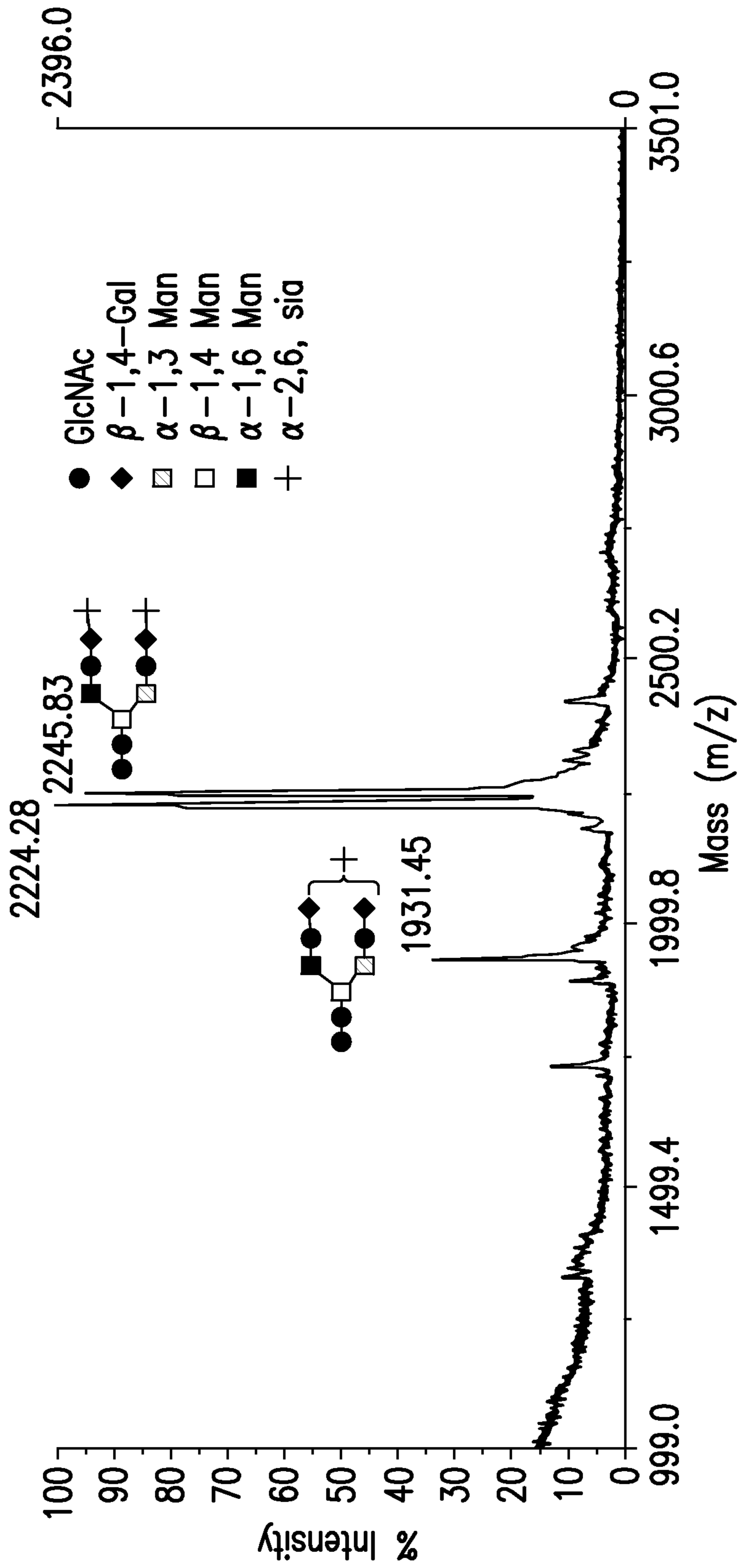


FIG.8

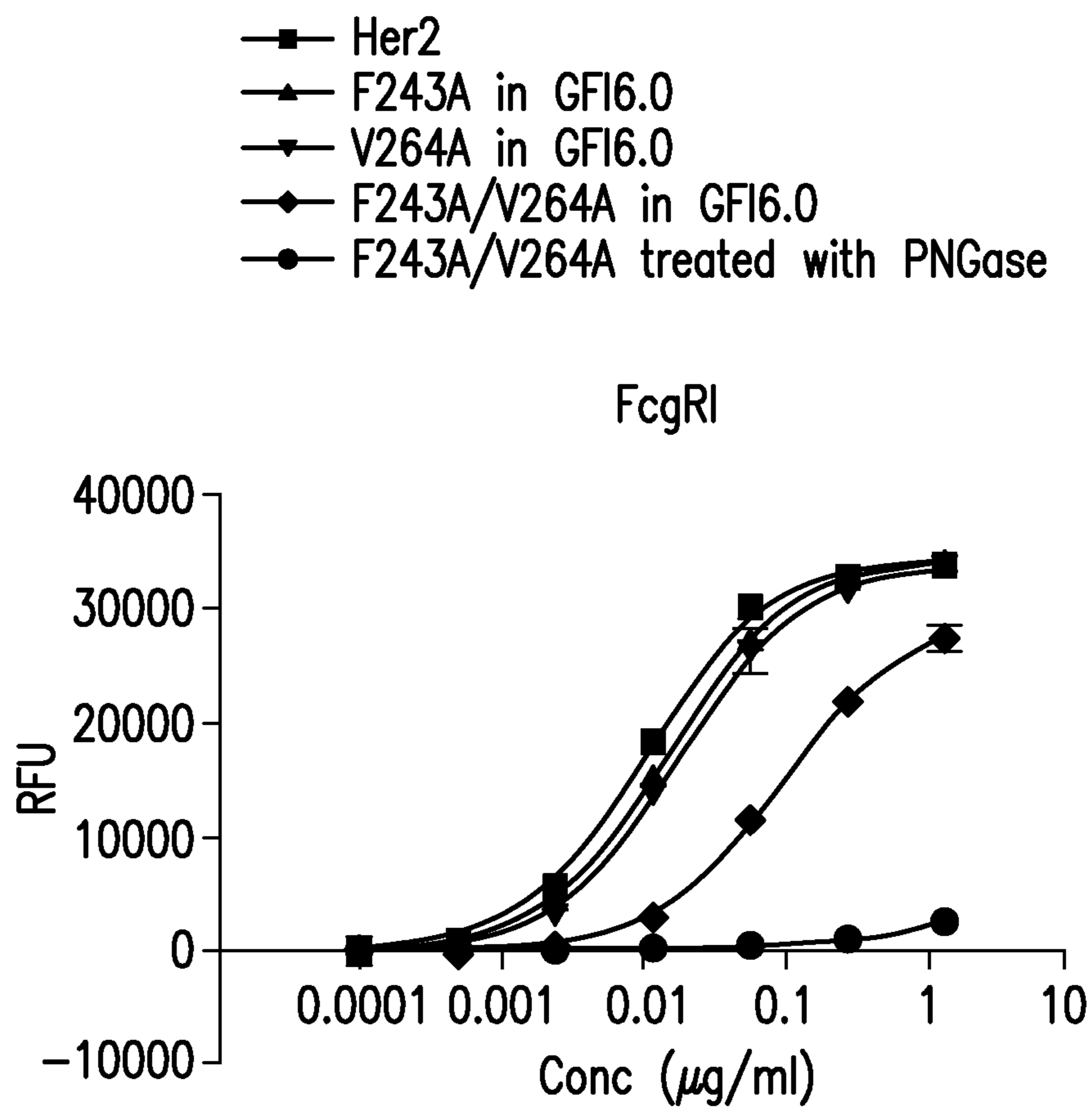


FIG.9A

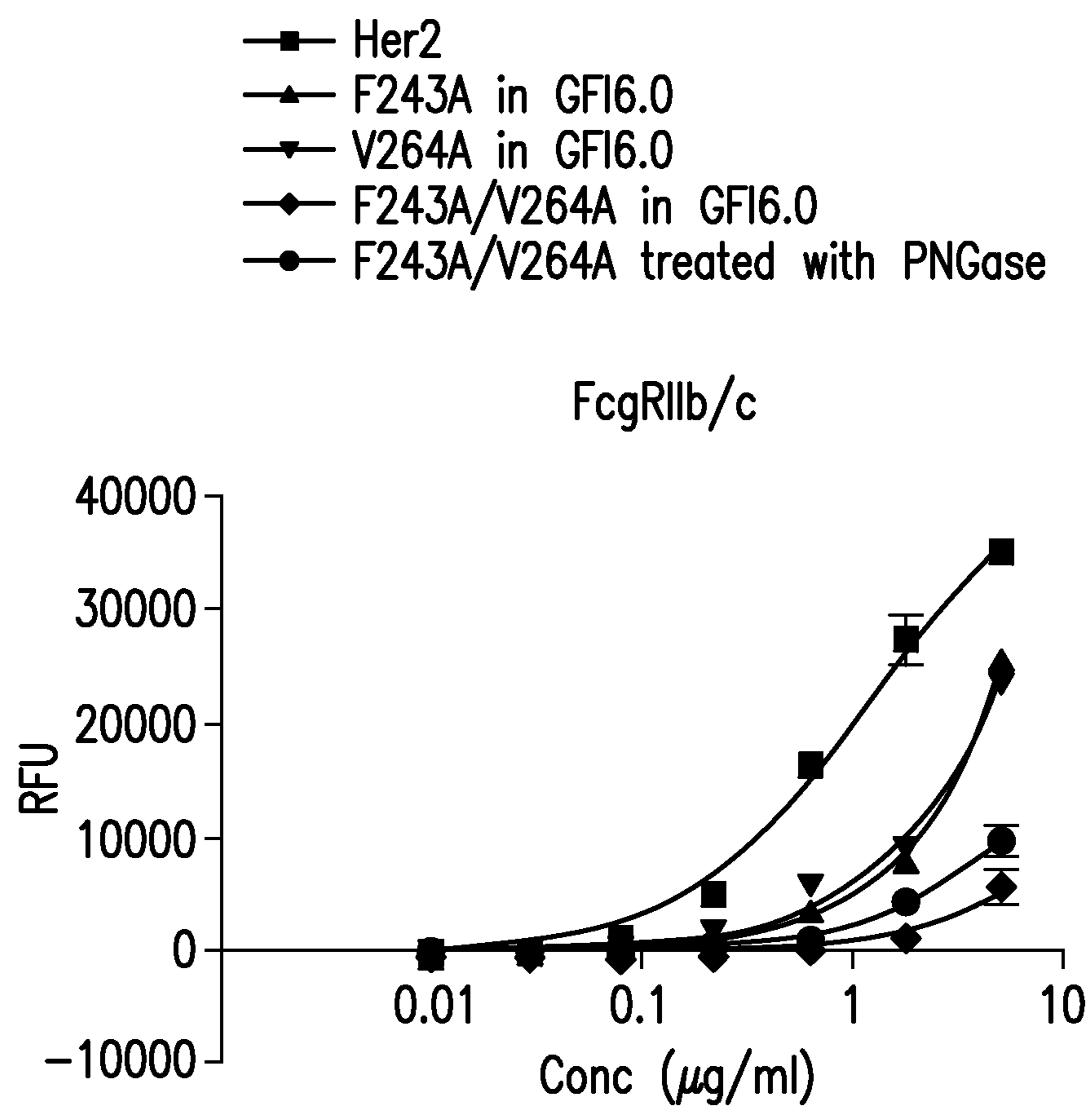


FIG.9B

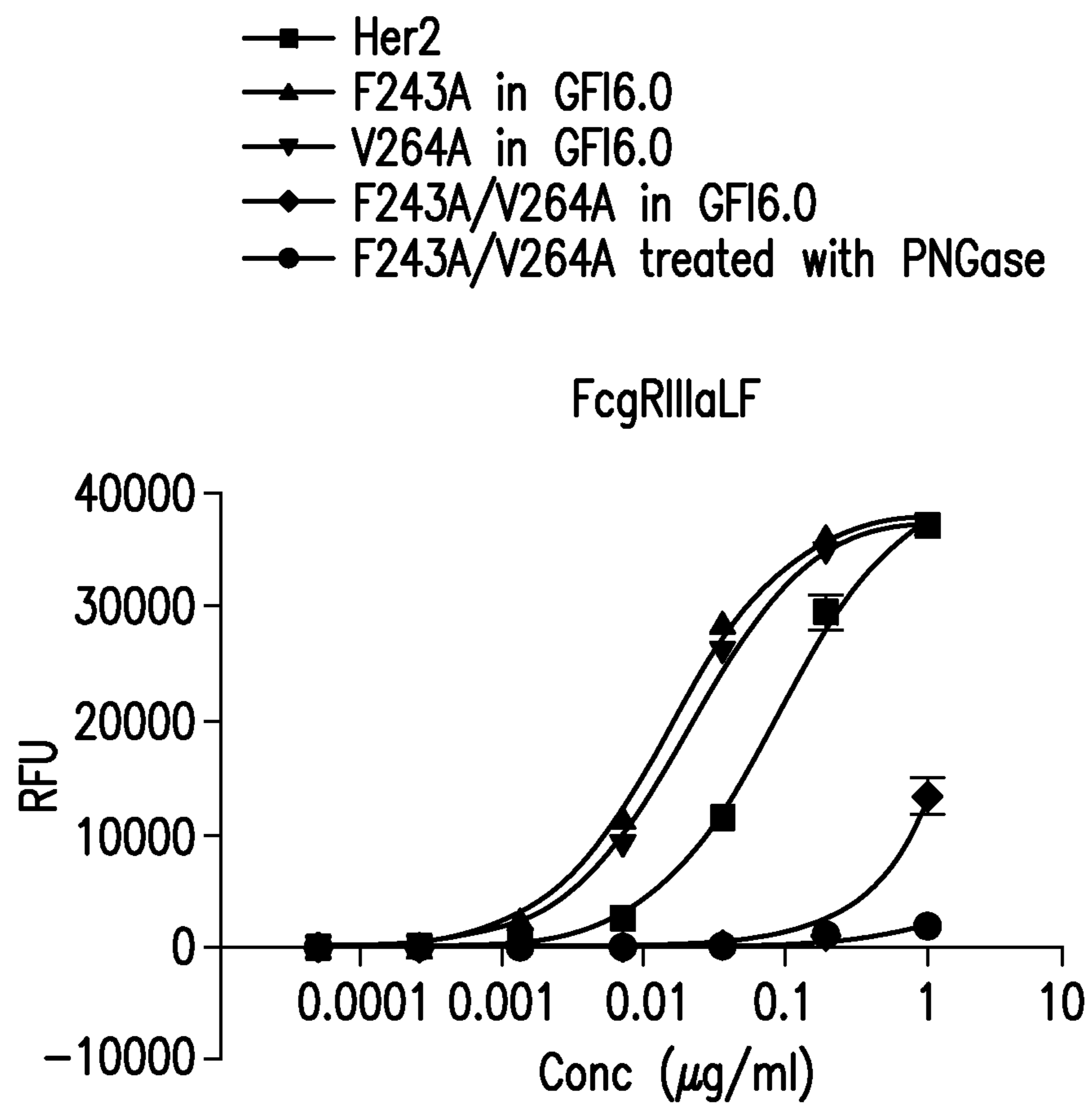


FIG.9C

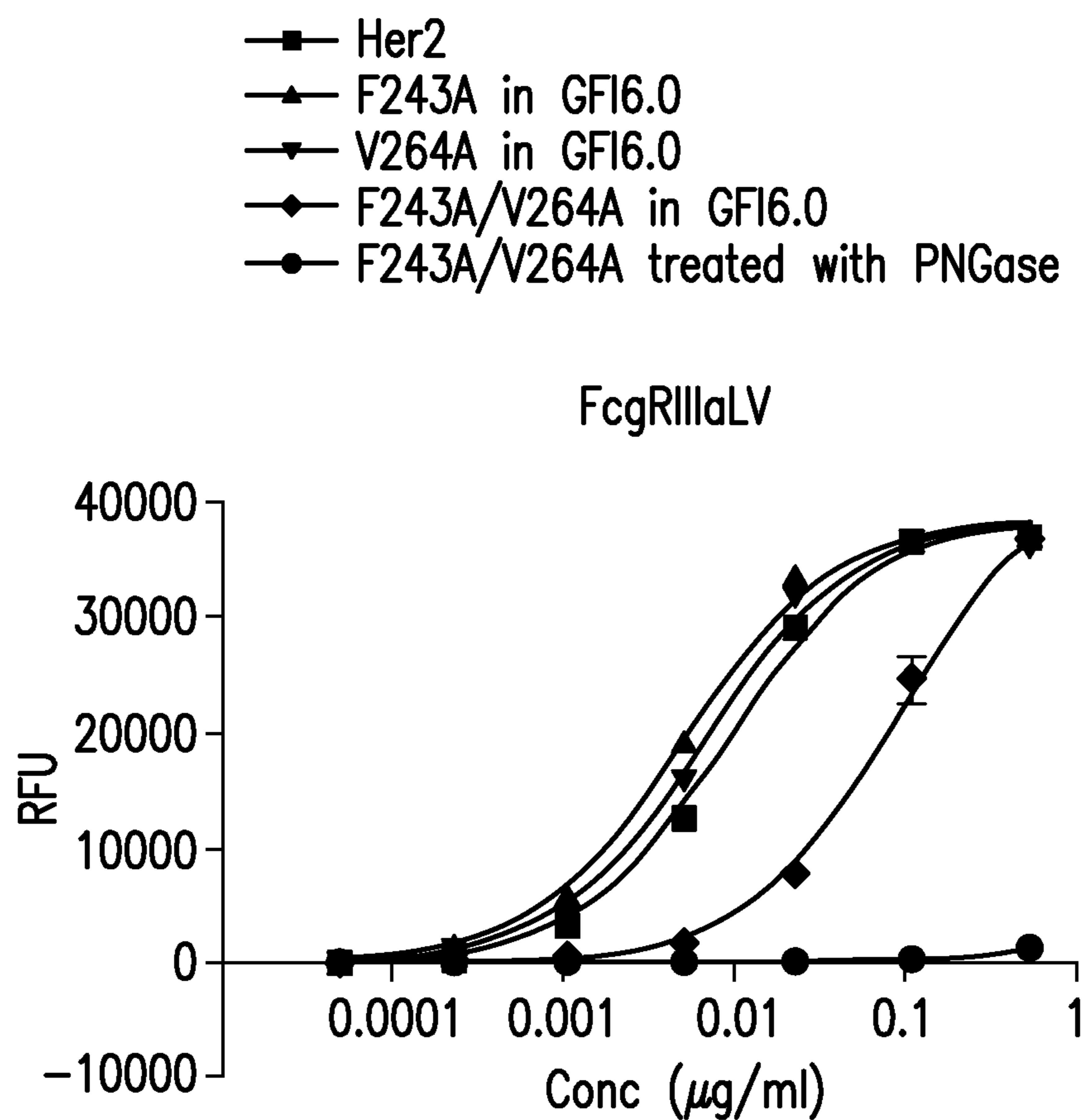


FIG.9D

- ◇— anti-CD20 antibody
- Her2
- ▲— F243A in GF16.0
- ▼— V264A in GF16.0
- ◆— F243A/V264A in GF16.0
- F243A/V264A in GF16.0 treated with PNGase

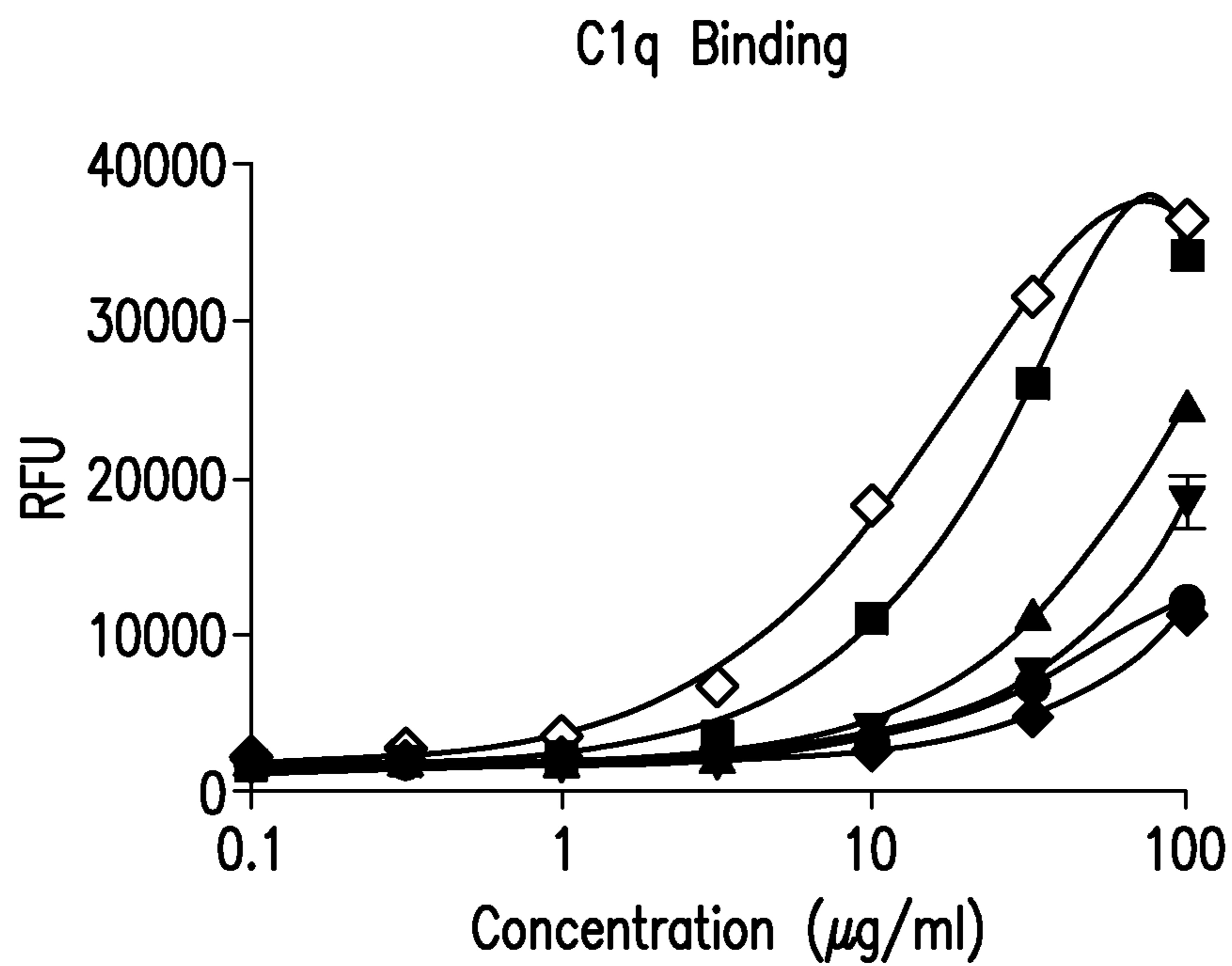


FIG. 10

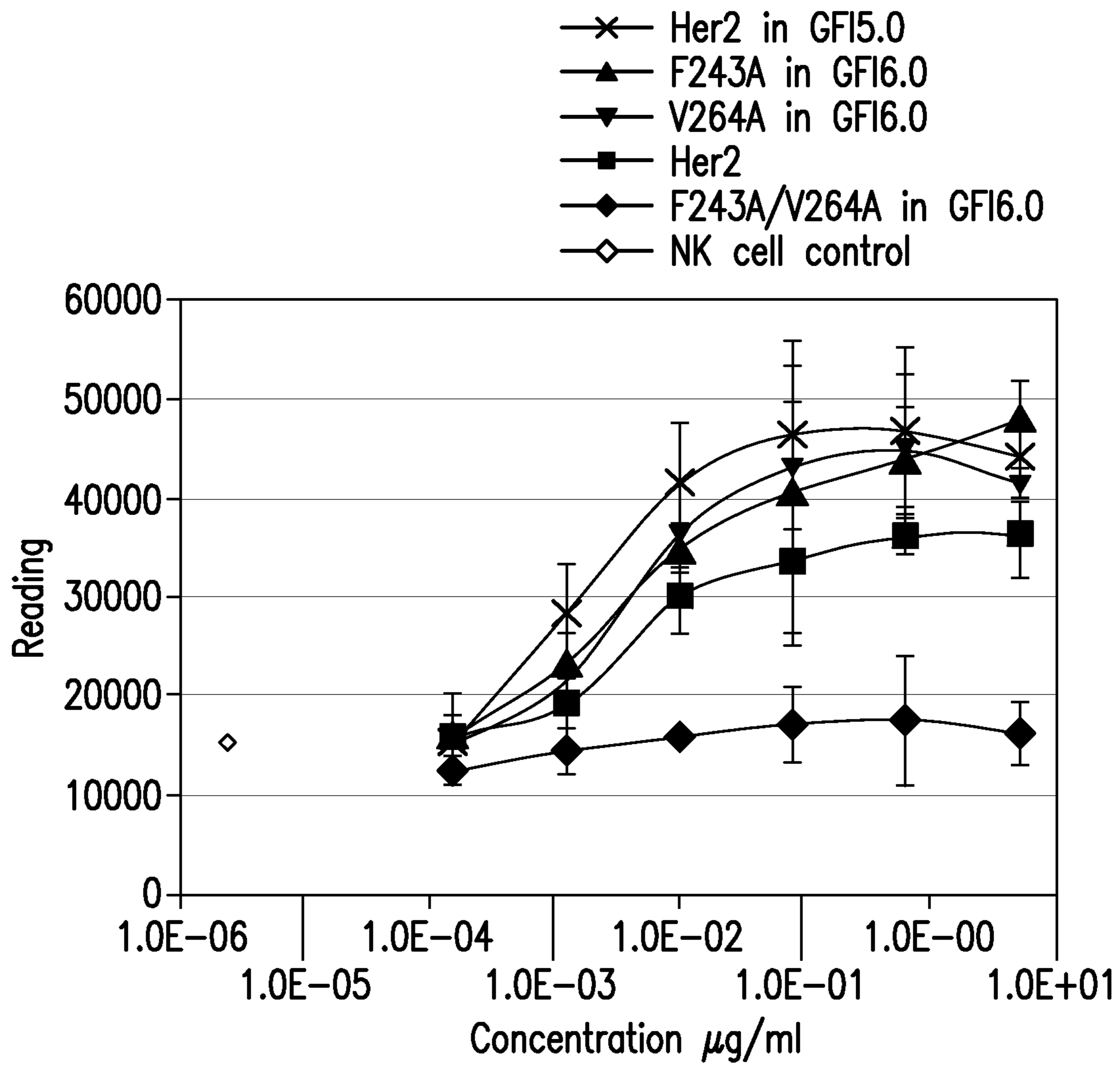


FIG. 11



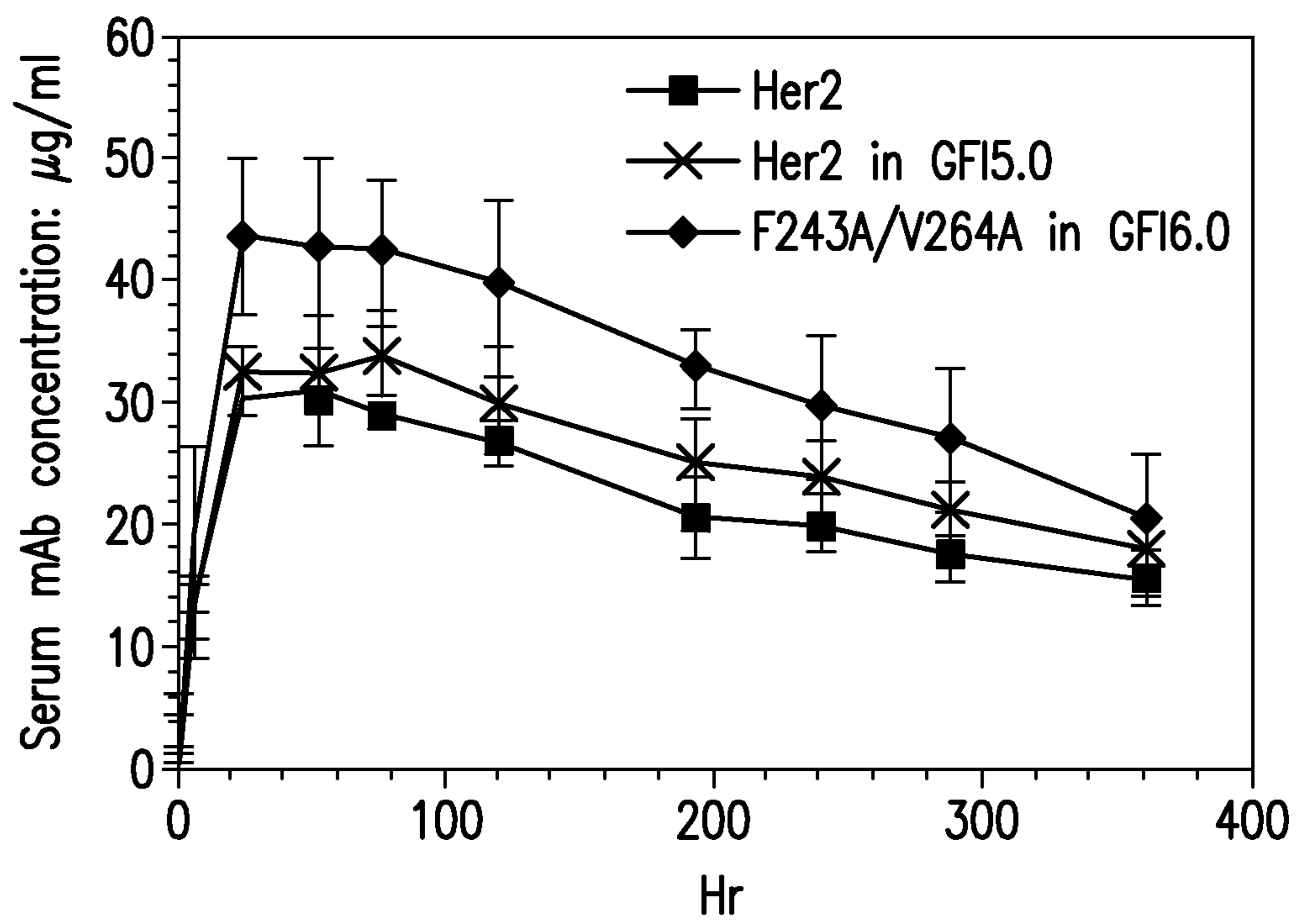


FIG. 12

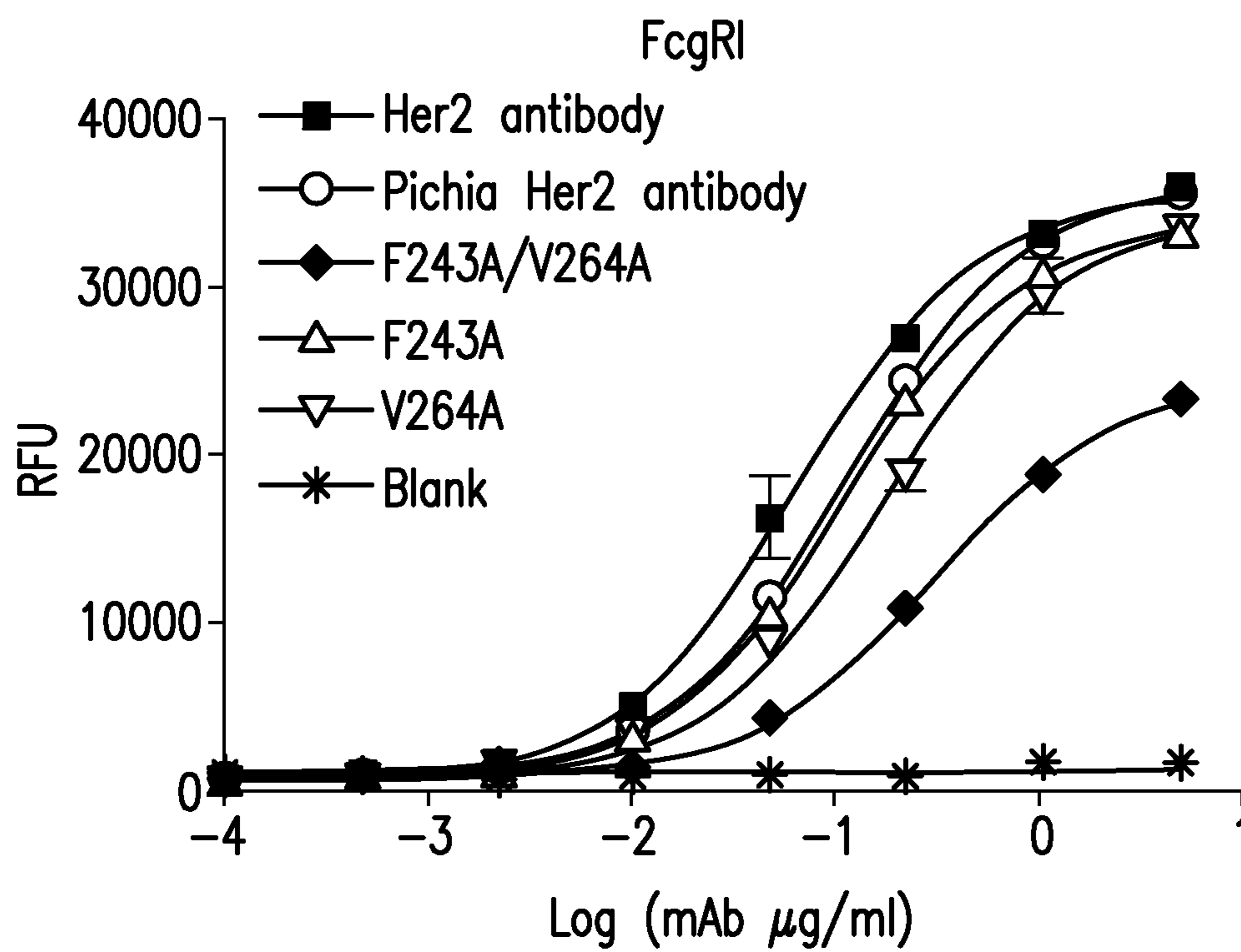


FIG. 13A

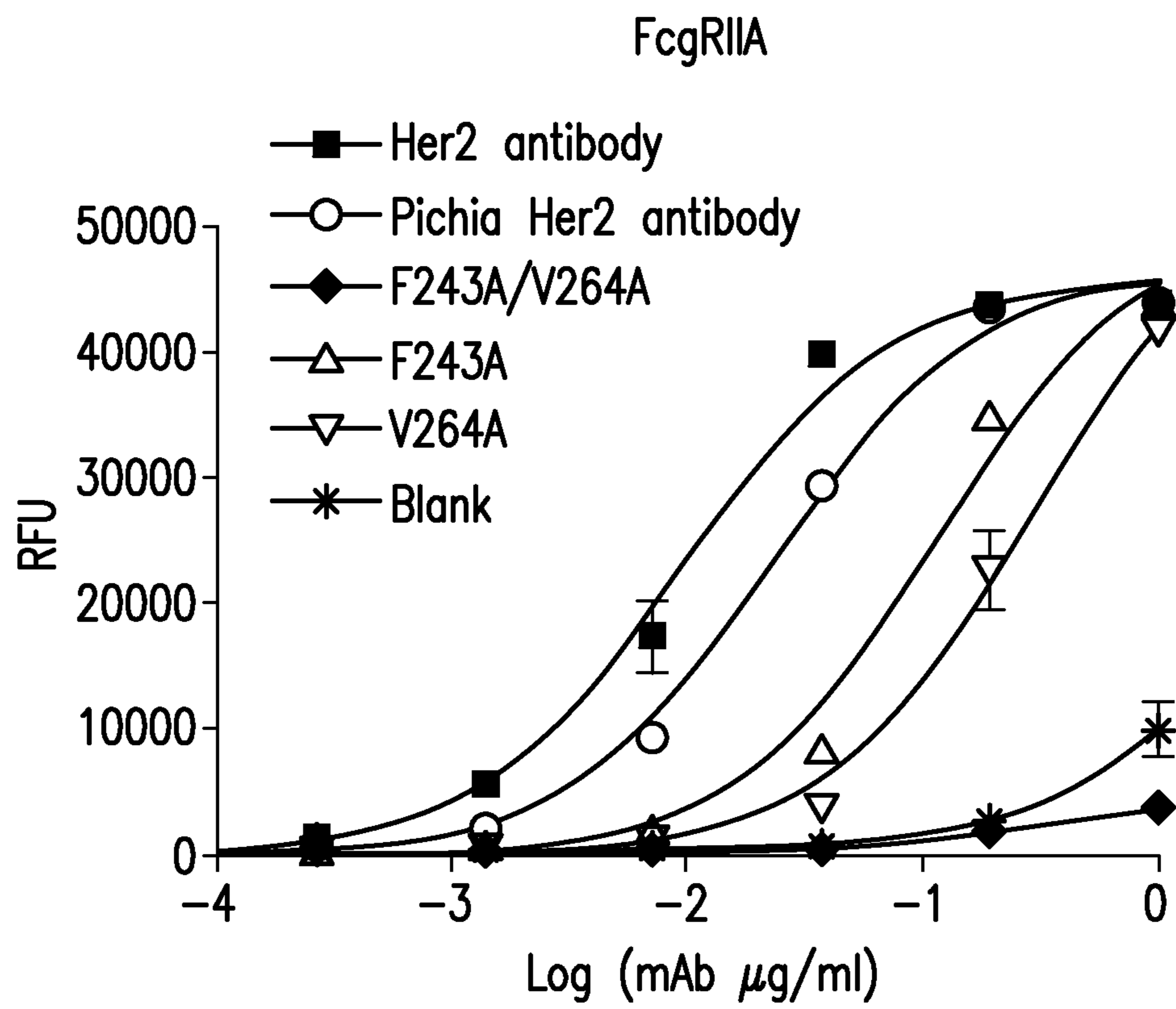


FIG. 13B

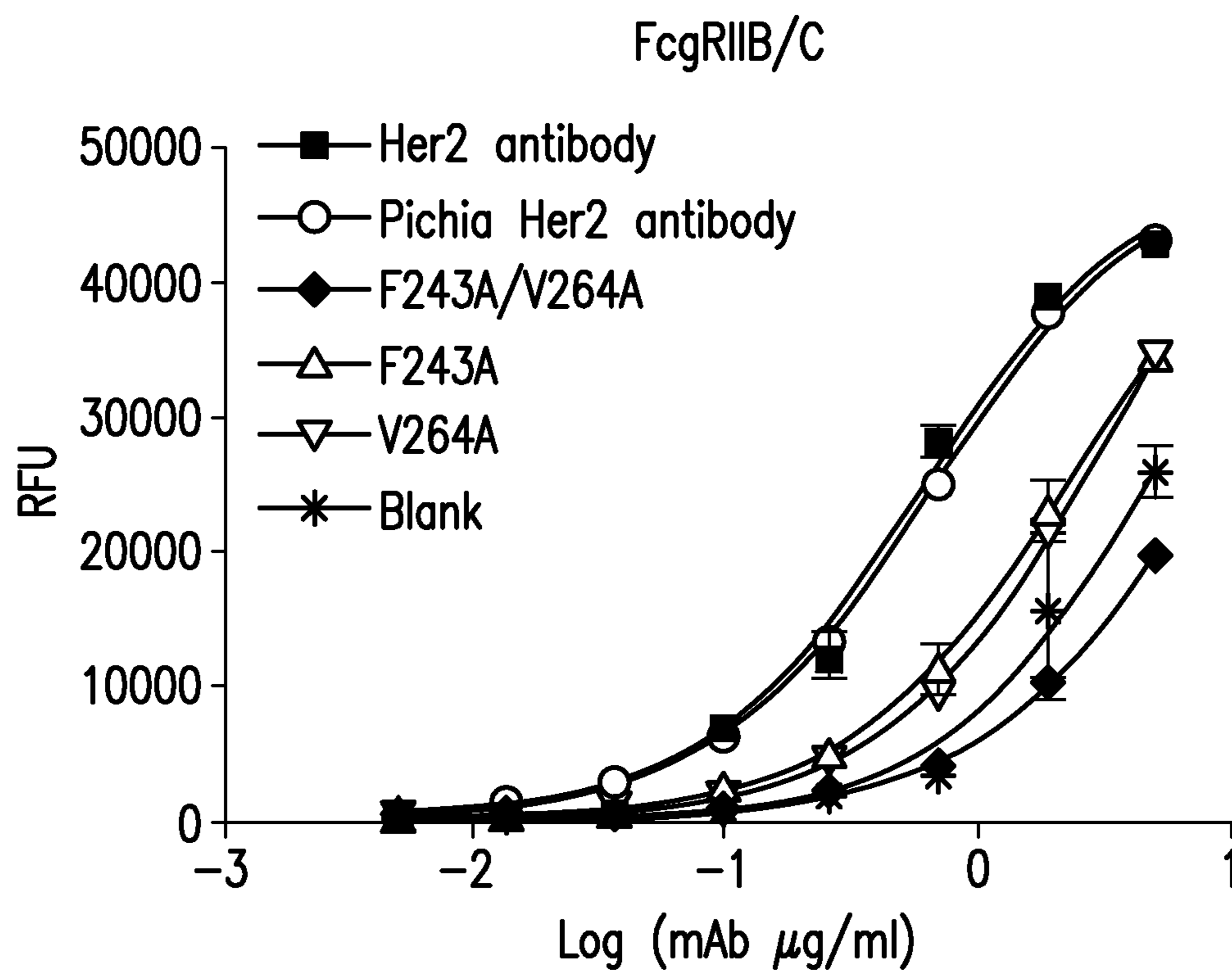


FIG. 13C

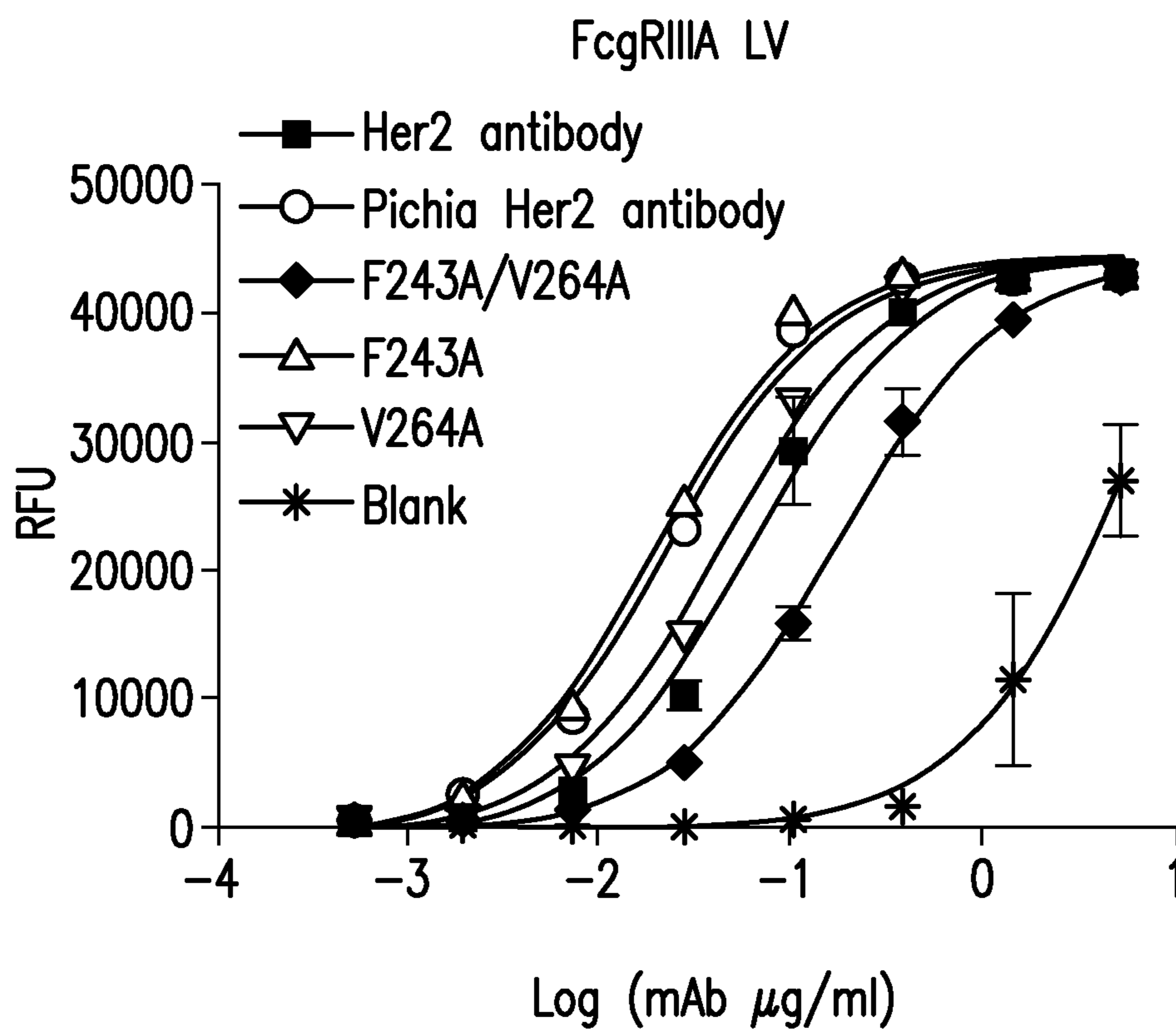


FIG. 13D

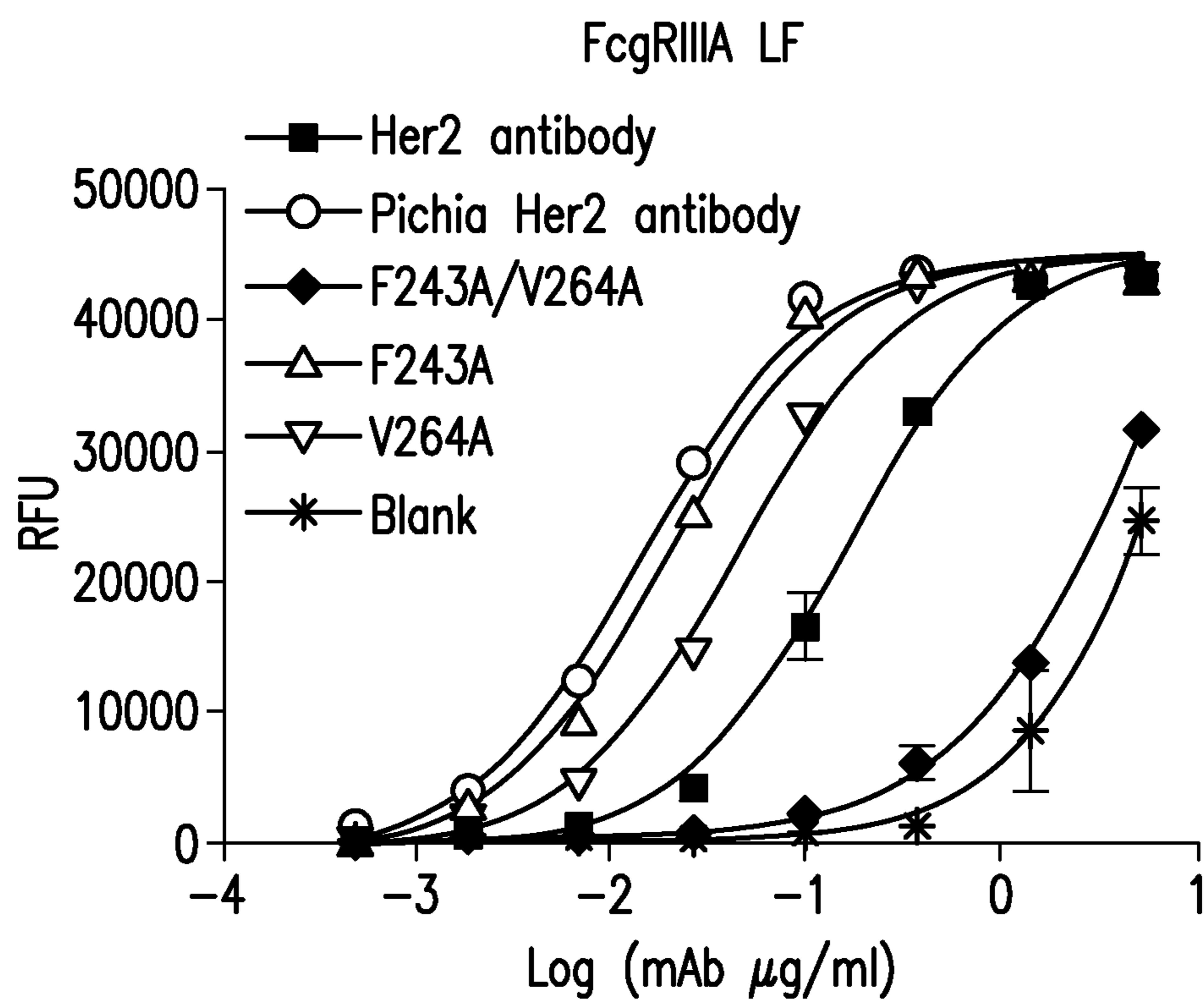
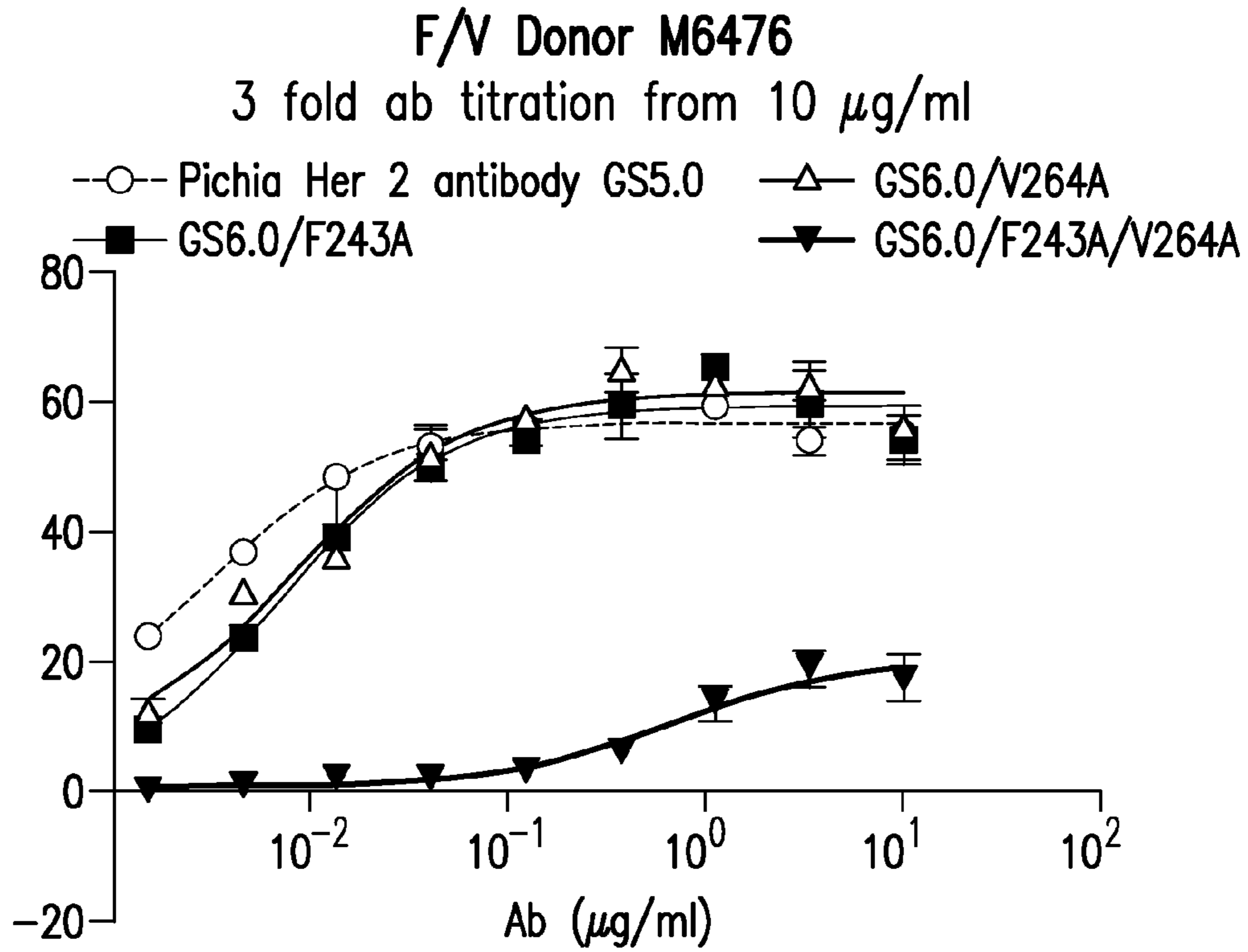
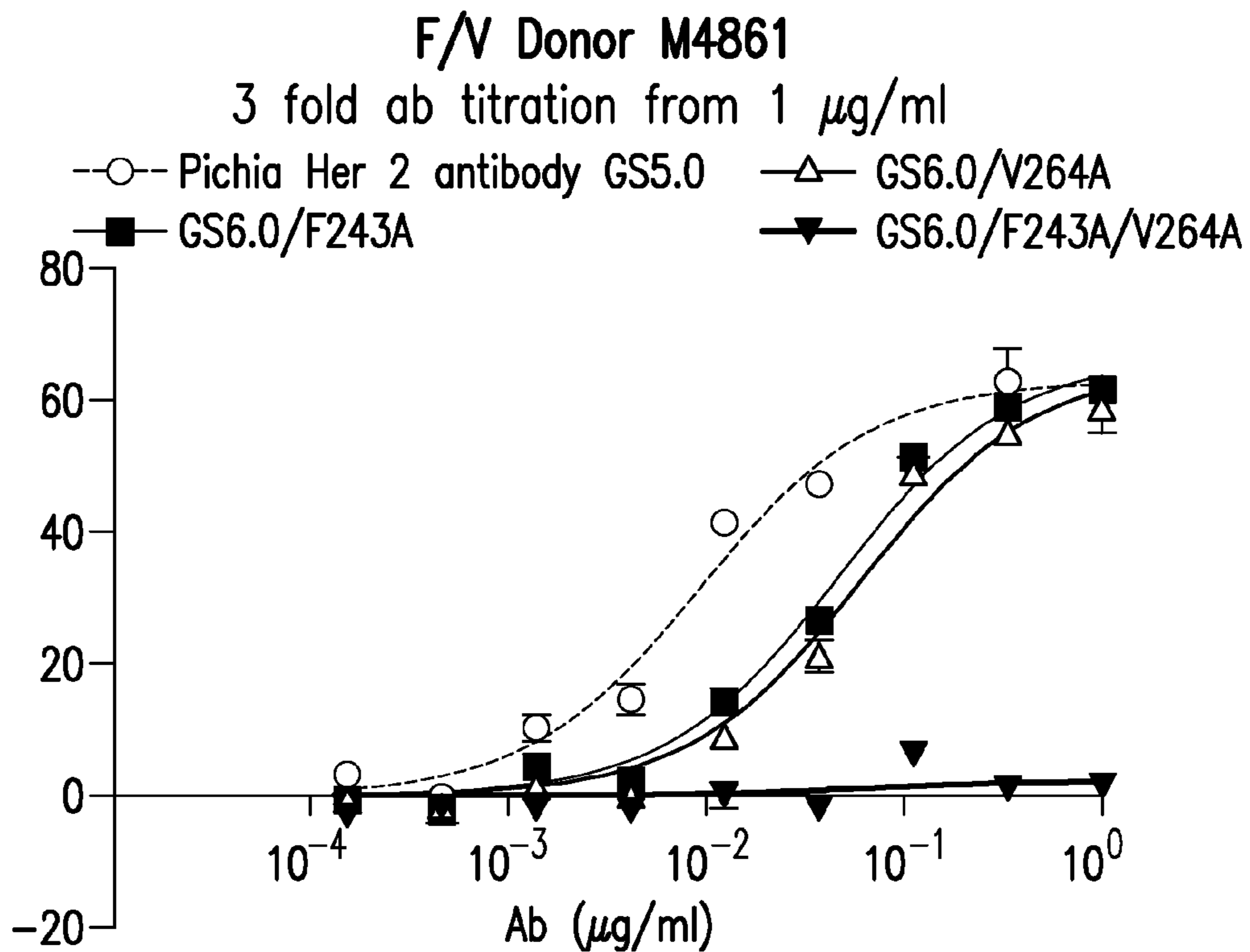


FIG. 13E



**FIG. 14A**



**FIG. 14B**

F/F Donor M5633

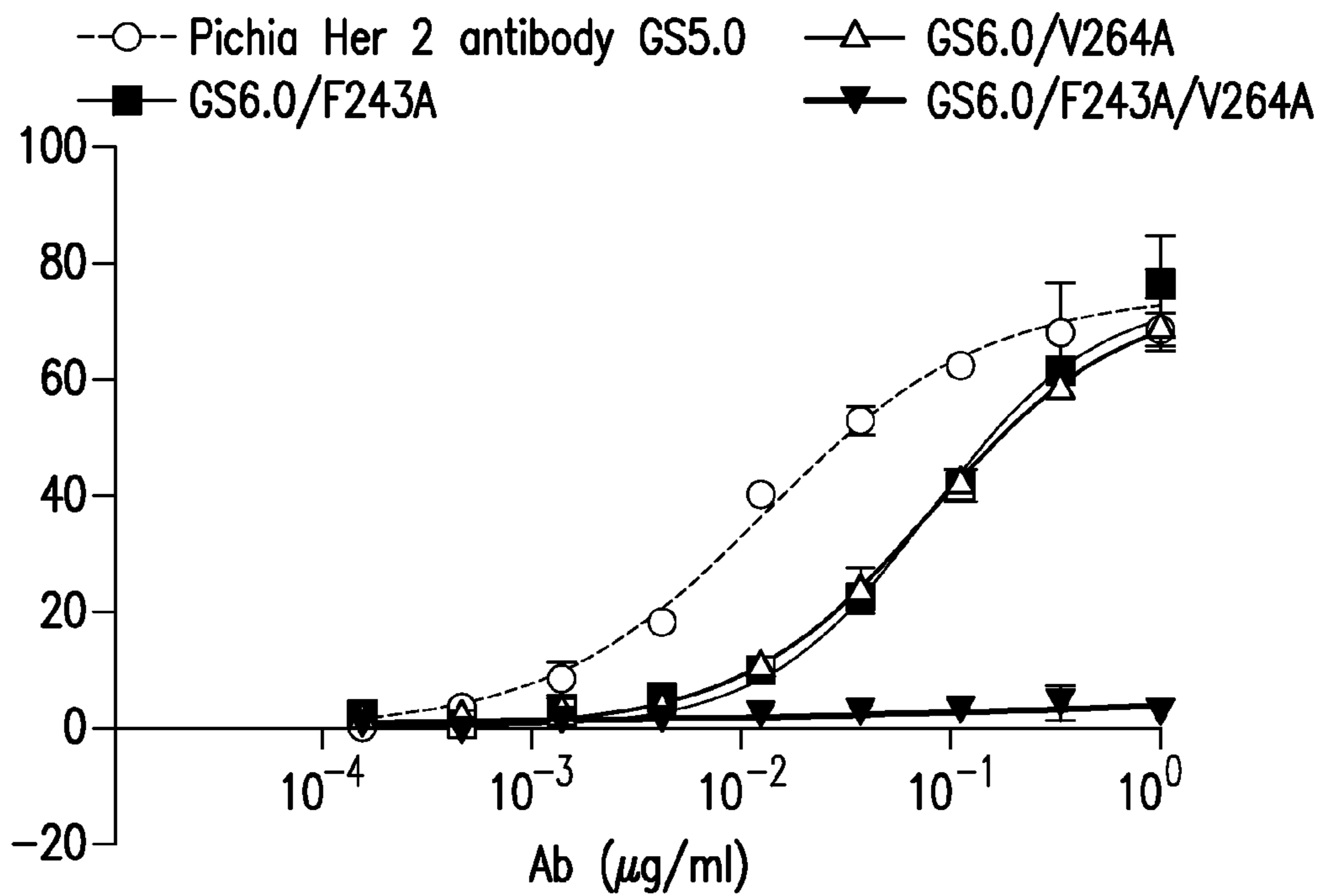


FIG. 14C

F/F Donor M6547

3 fold ab titration from 1 µg/ml

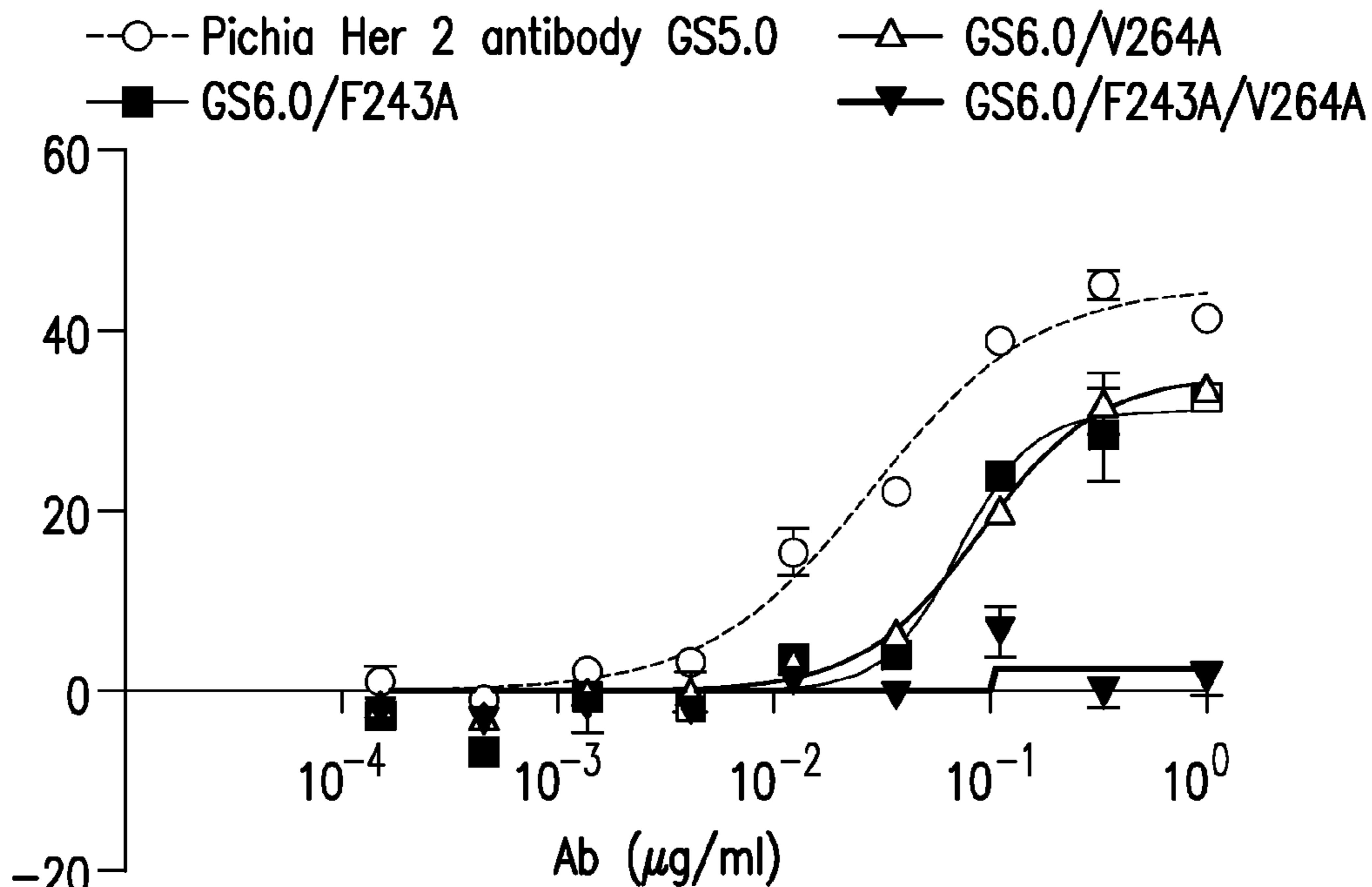
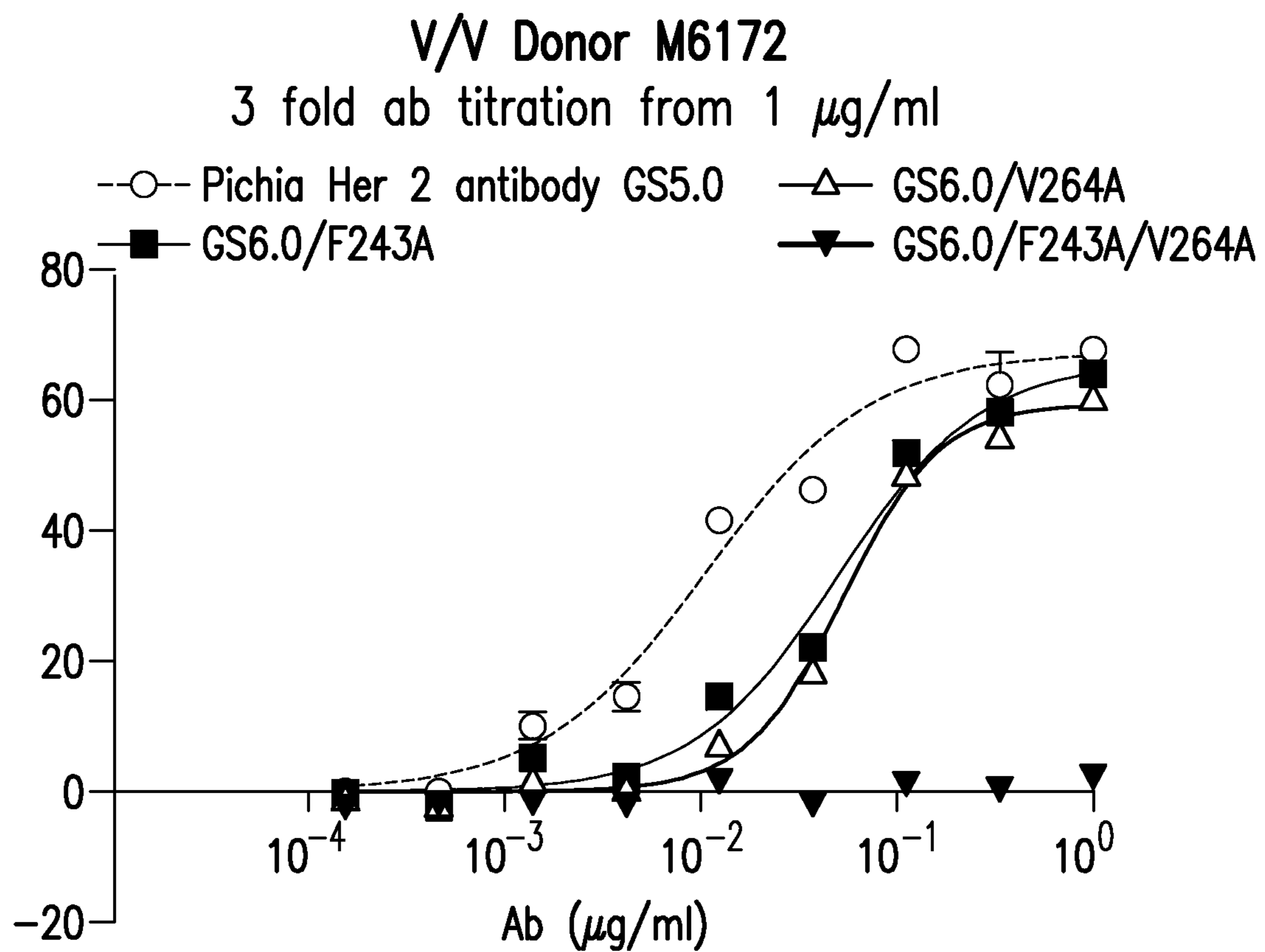


FIG. 14D





**FIG. 14E**

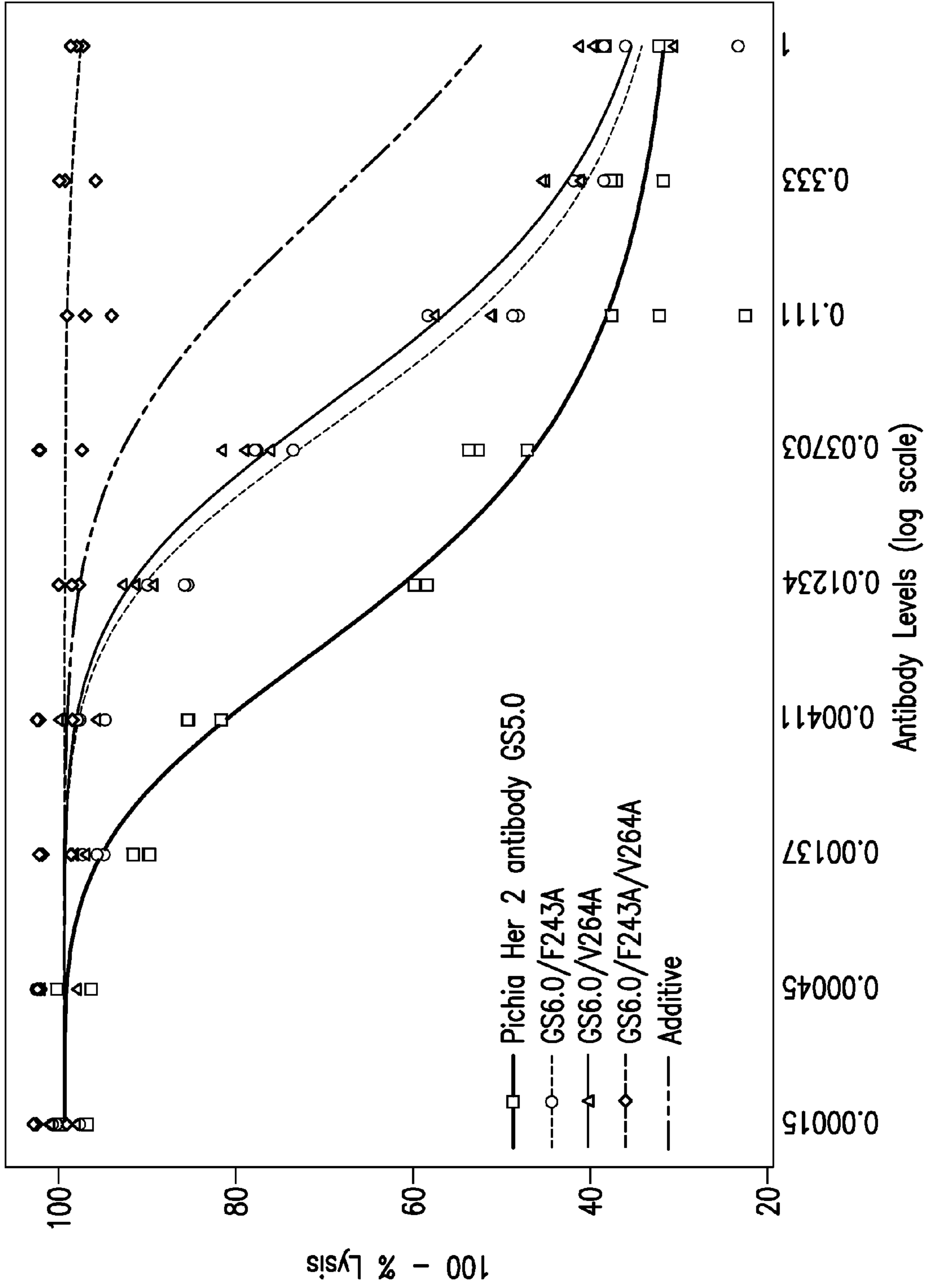
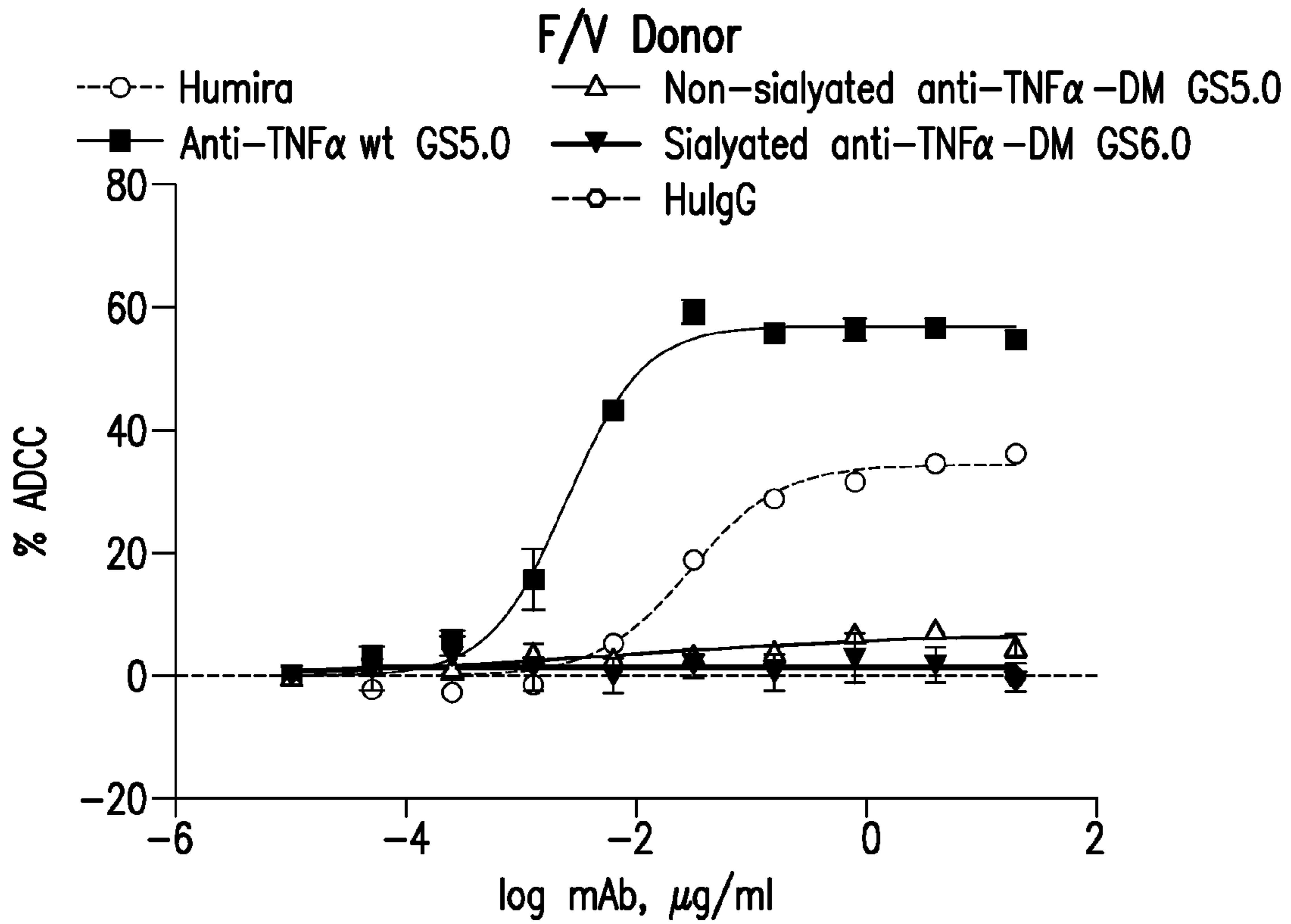
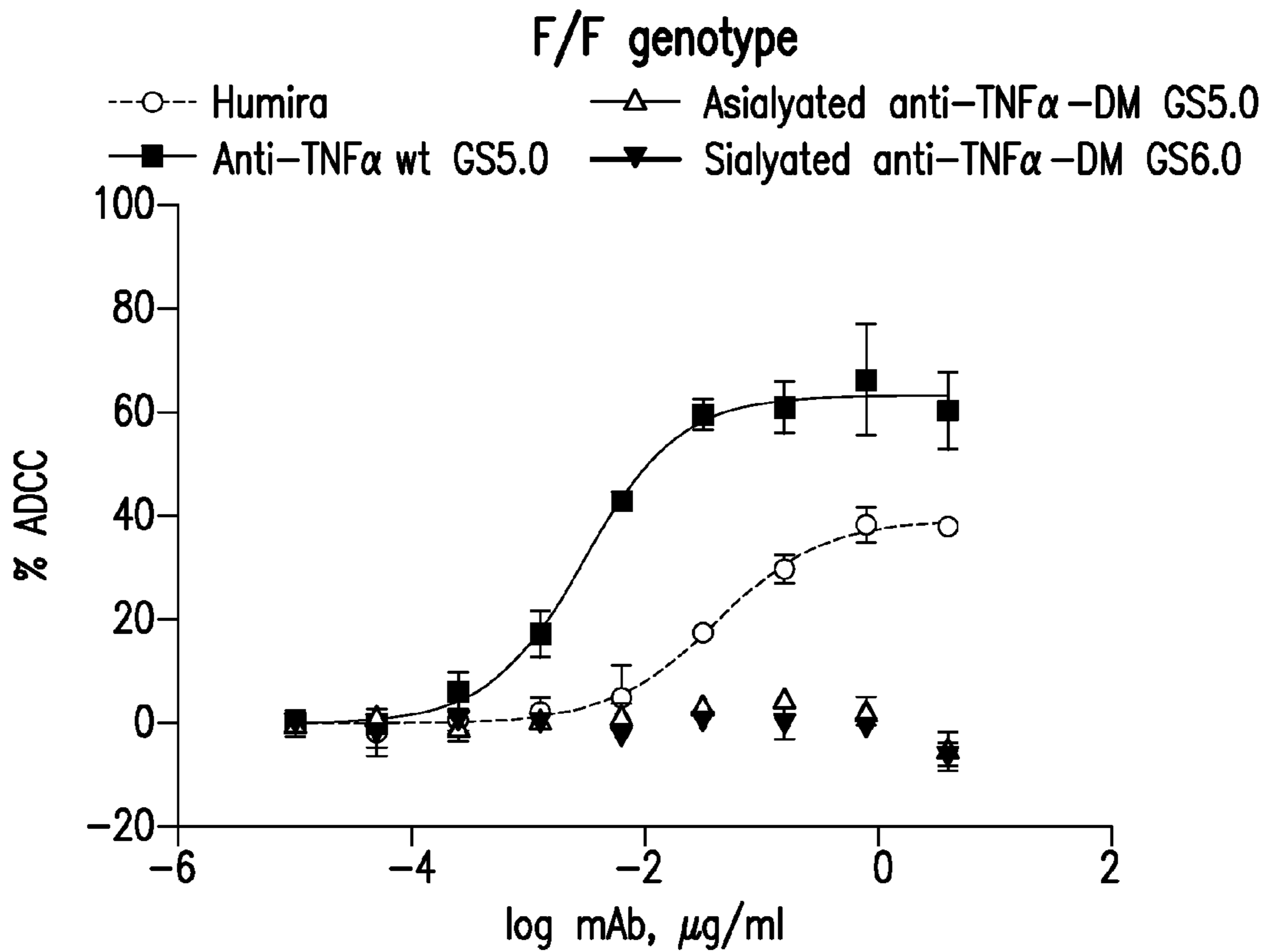


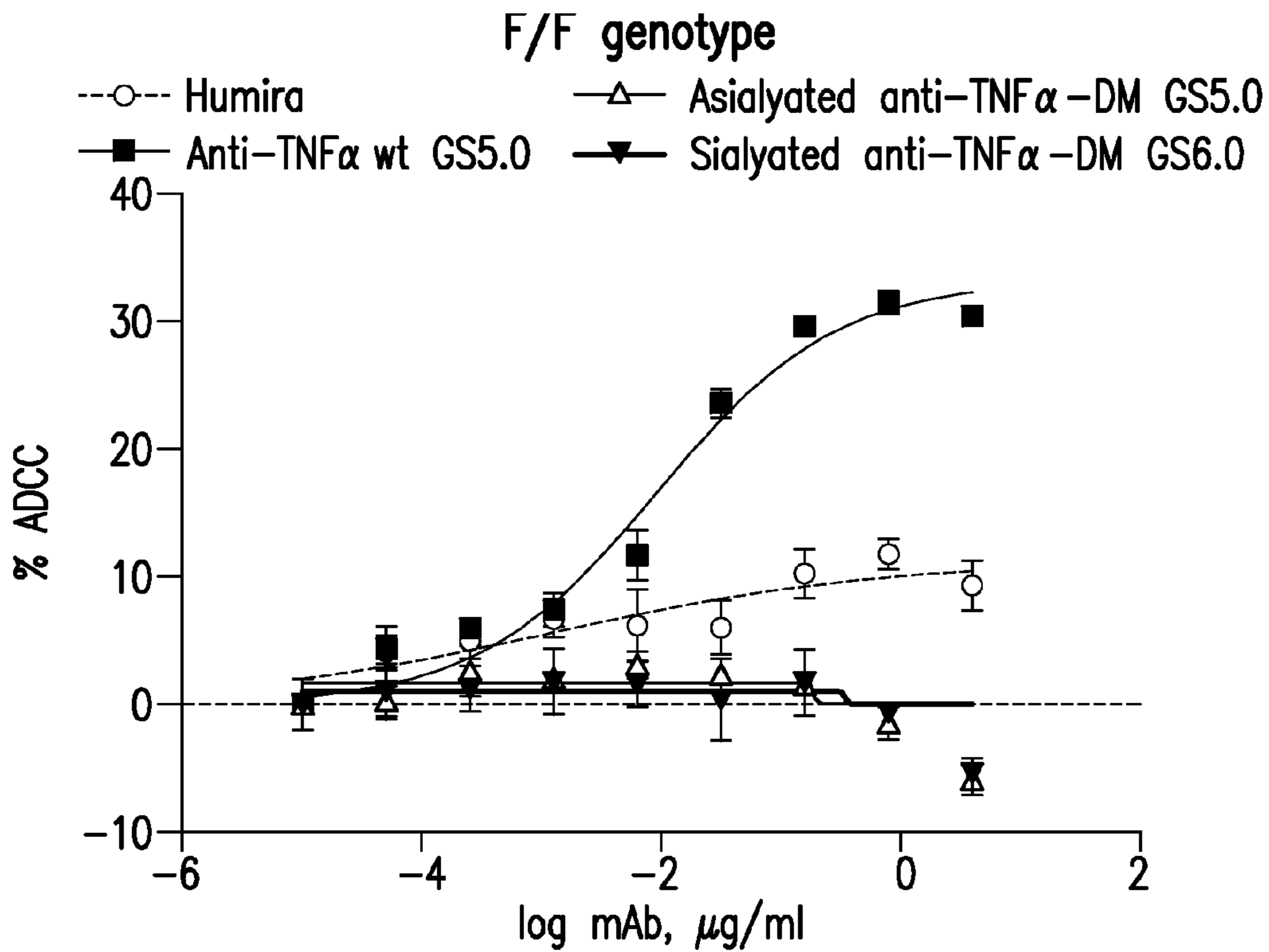
FIG. 15



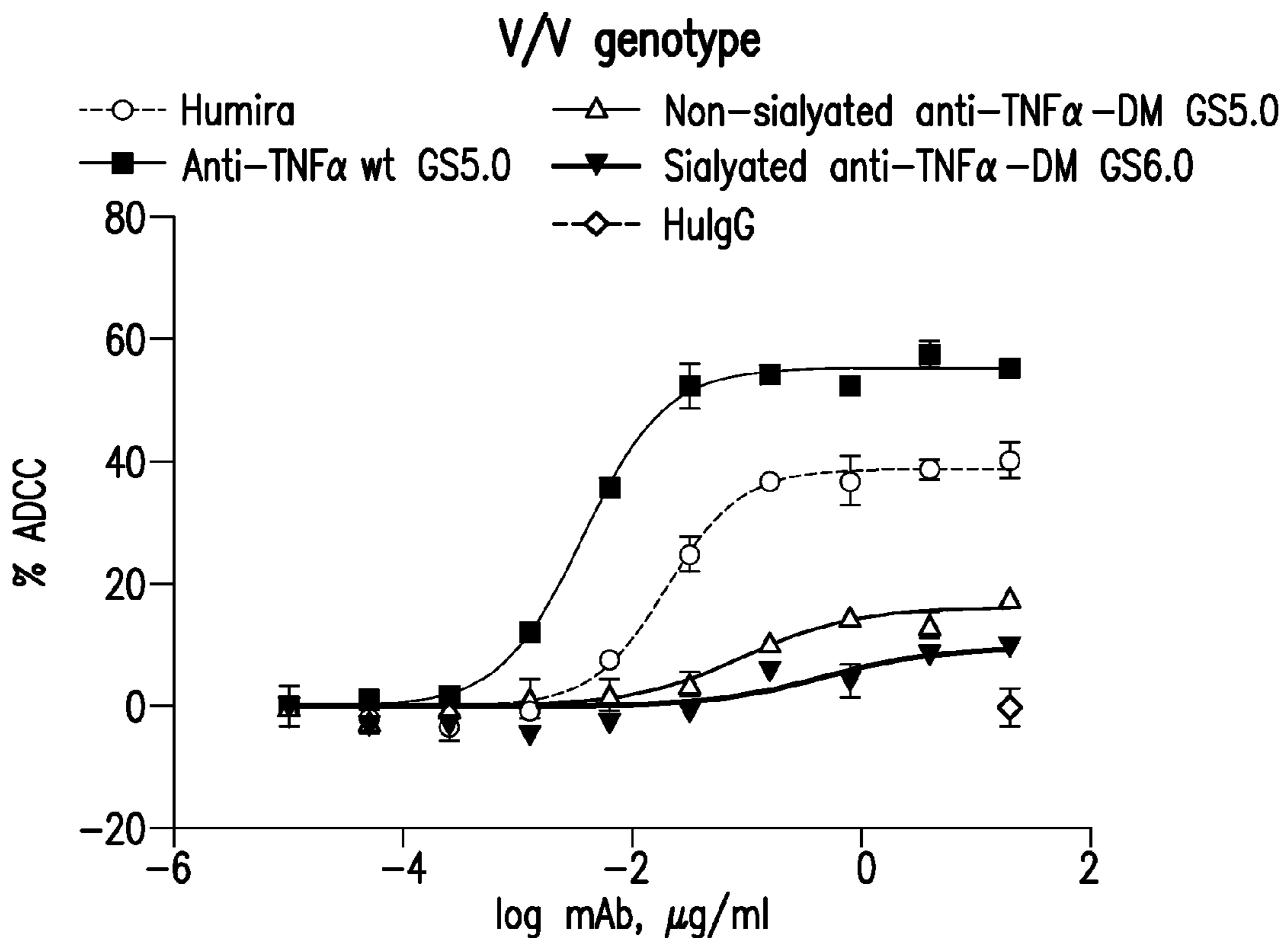
**FIG. 16A**



**FIG. 16B**



**FIG. 16C**



**FIG. 16D**

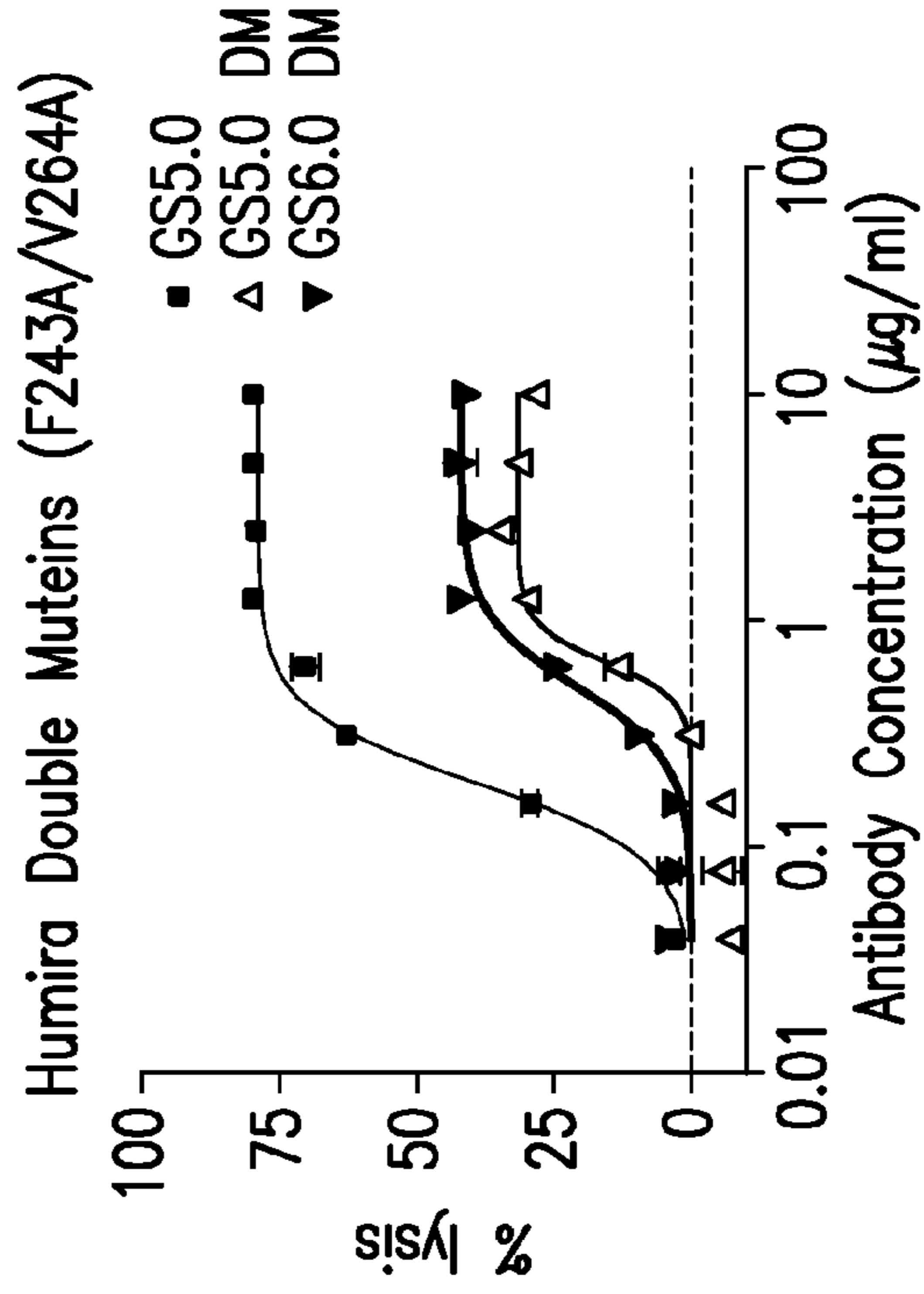
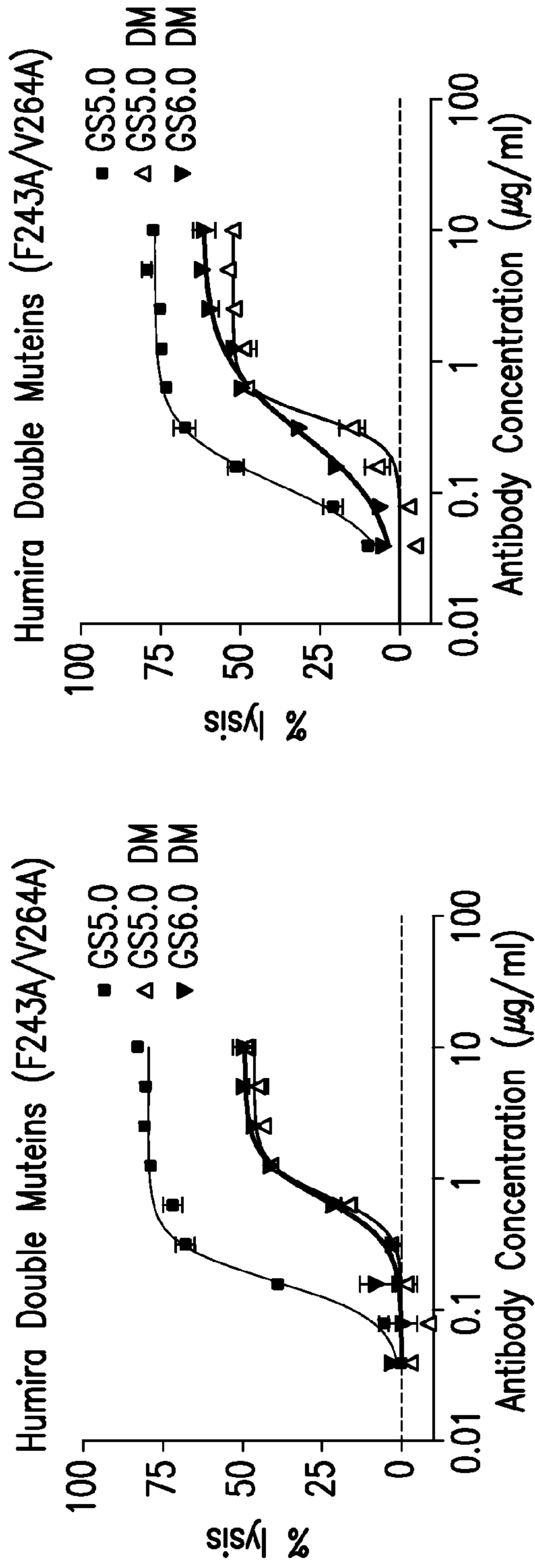


FIG. 17

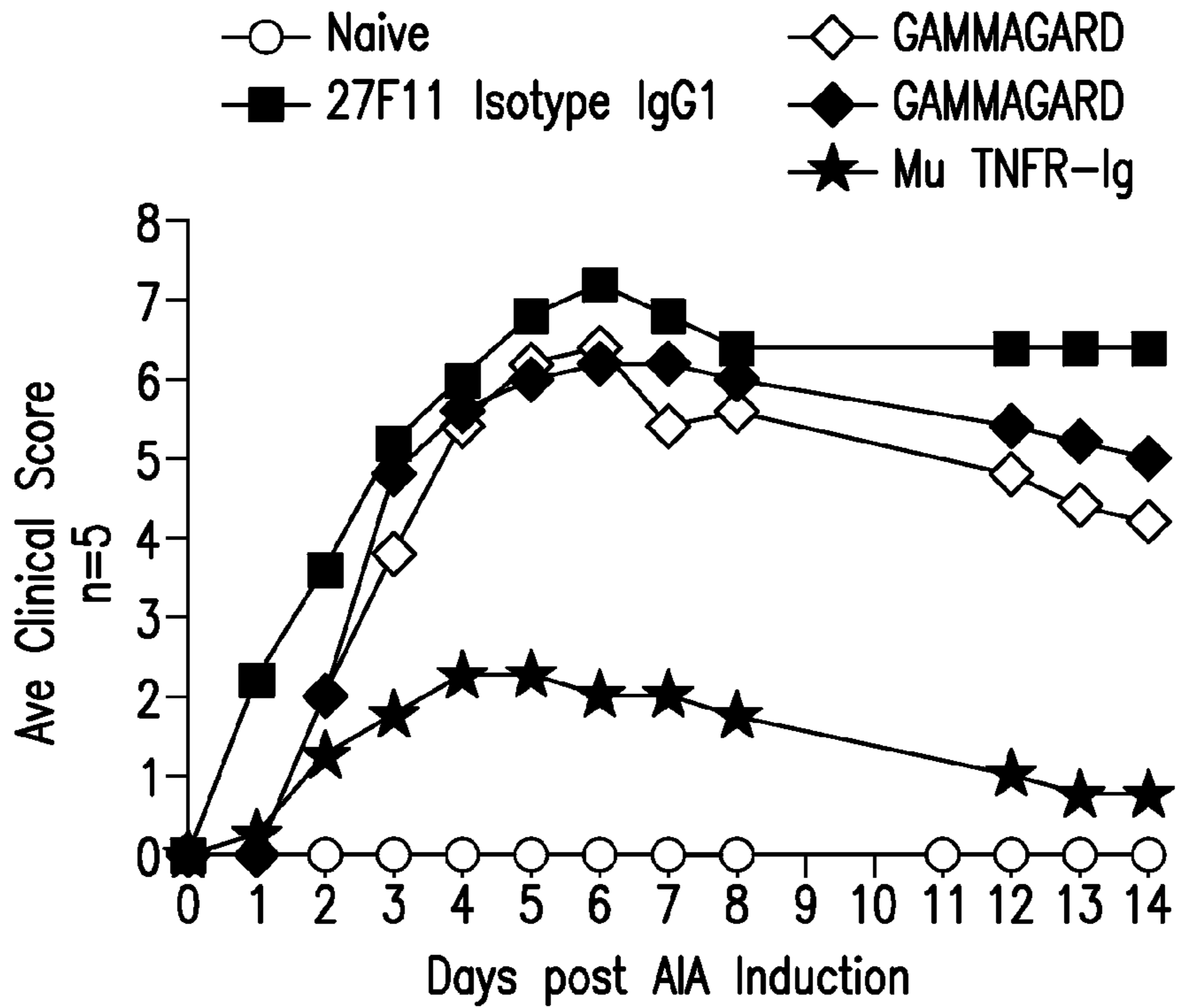


FIG. 18A

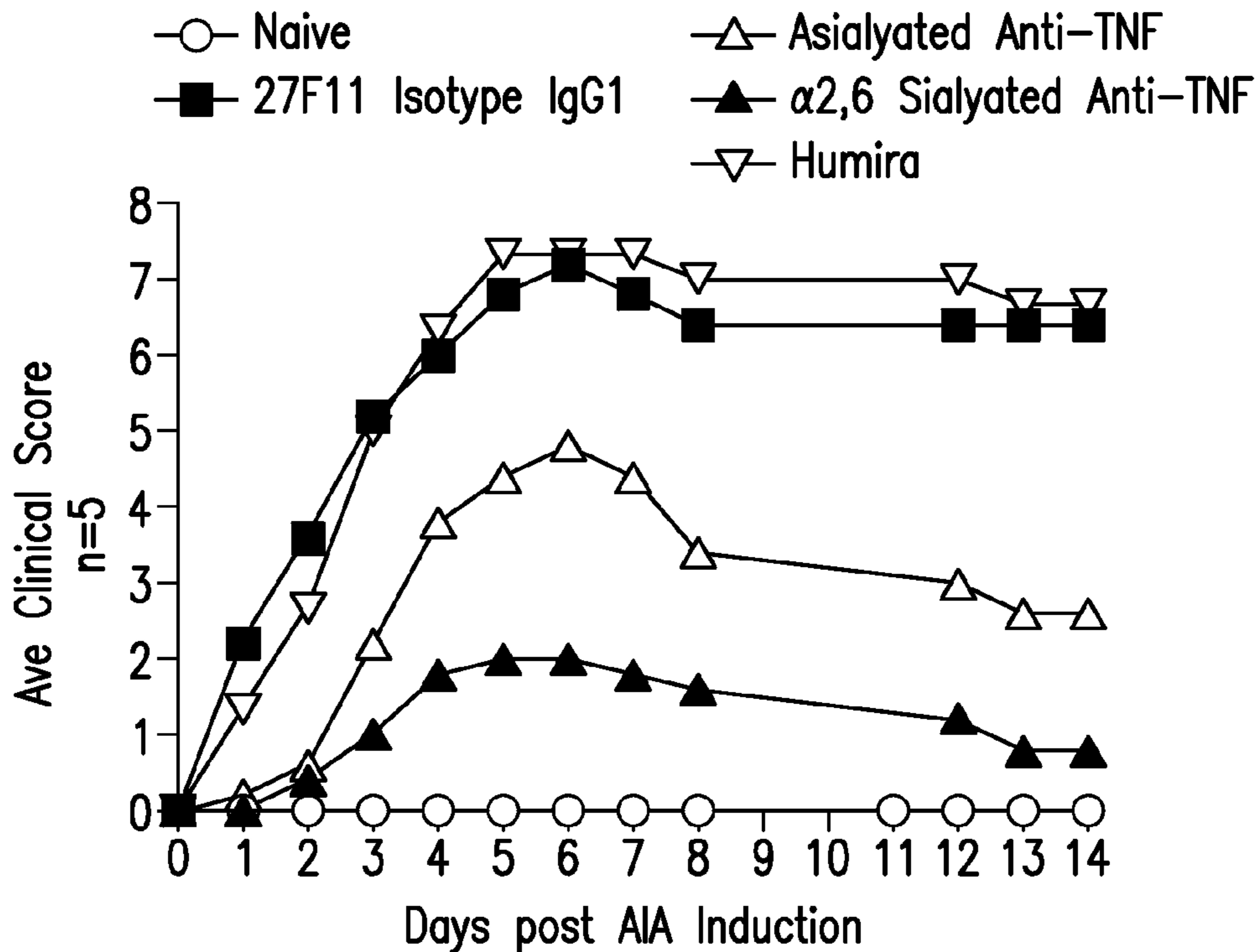


FIG. 18B

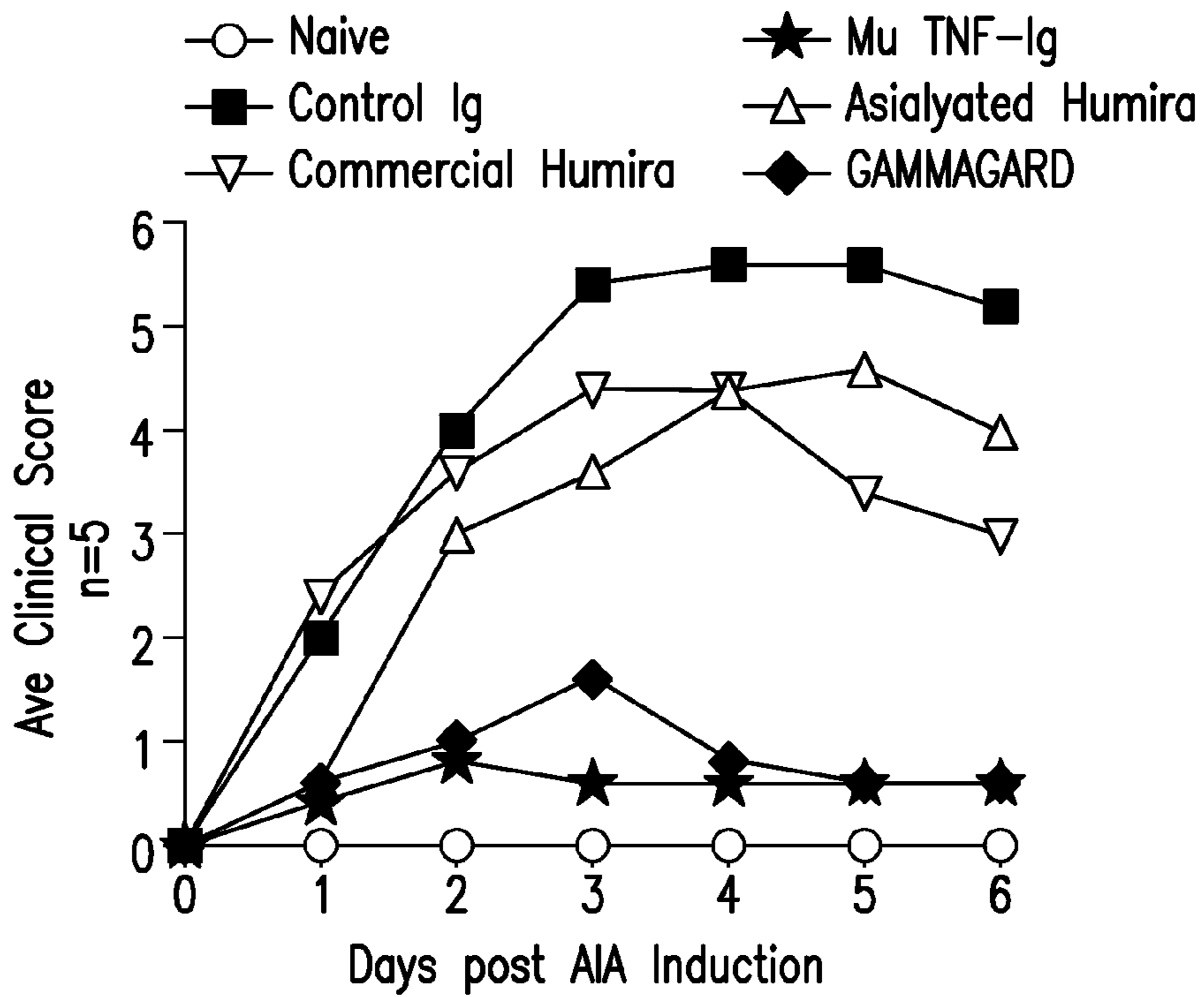


FIG. 19A

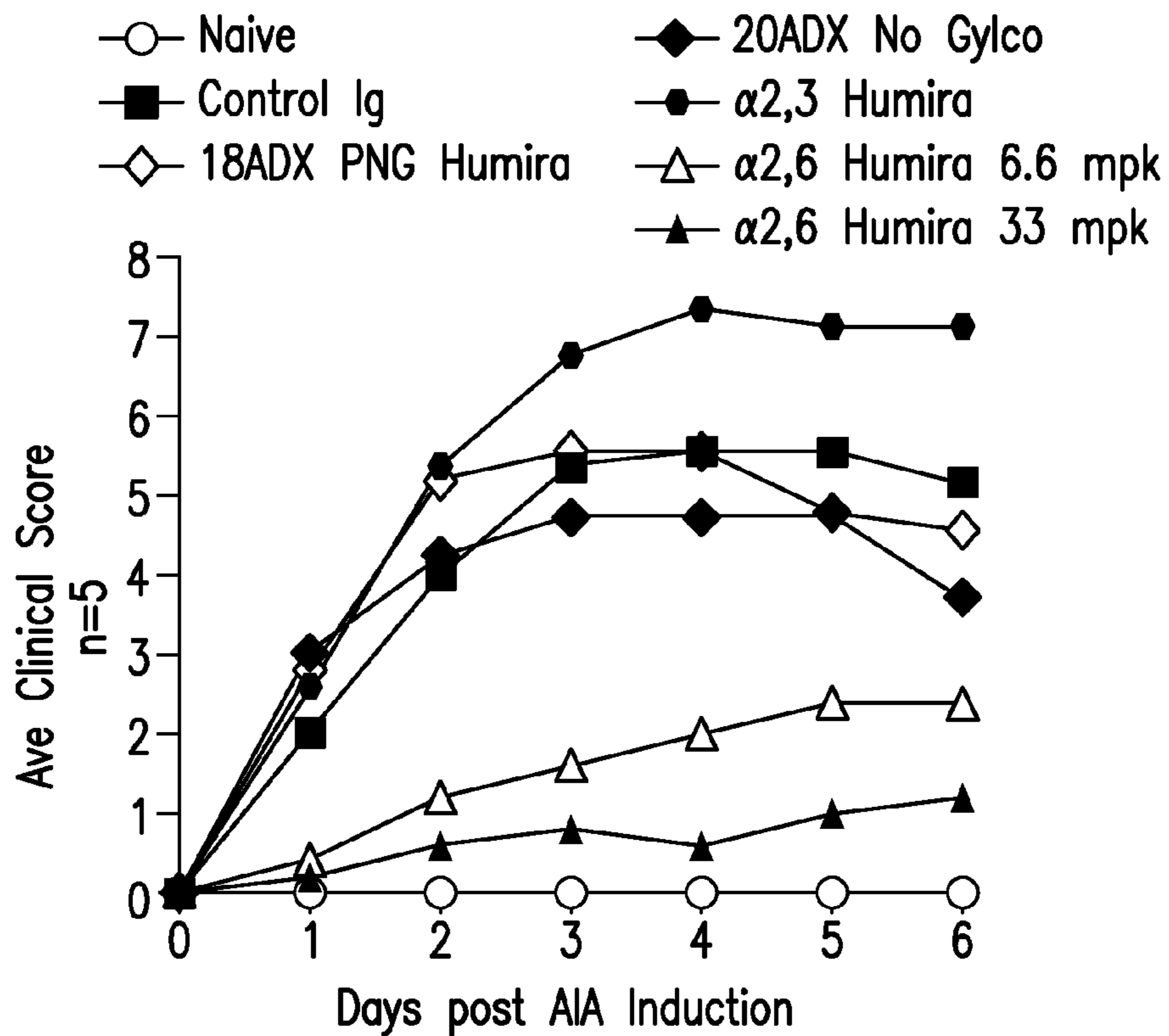


FIG. 19B

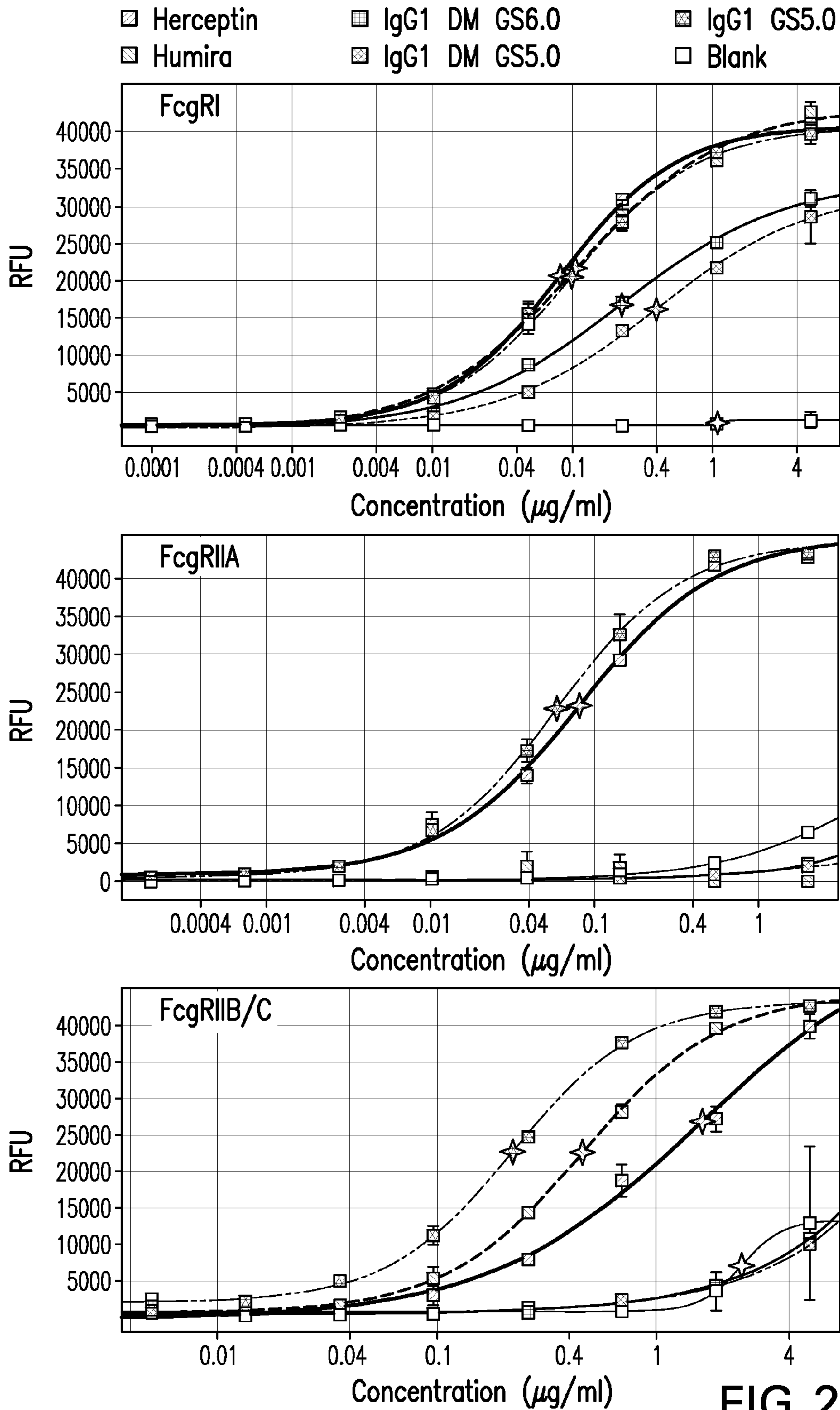


FIG.20-1



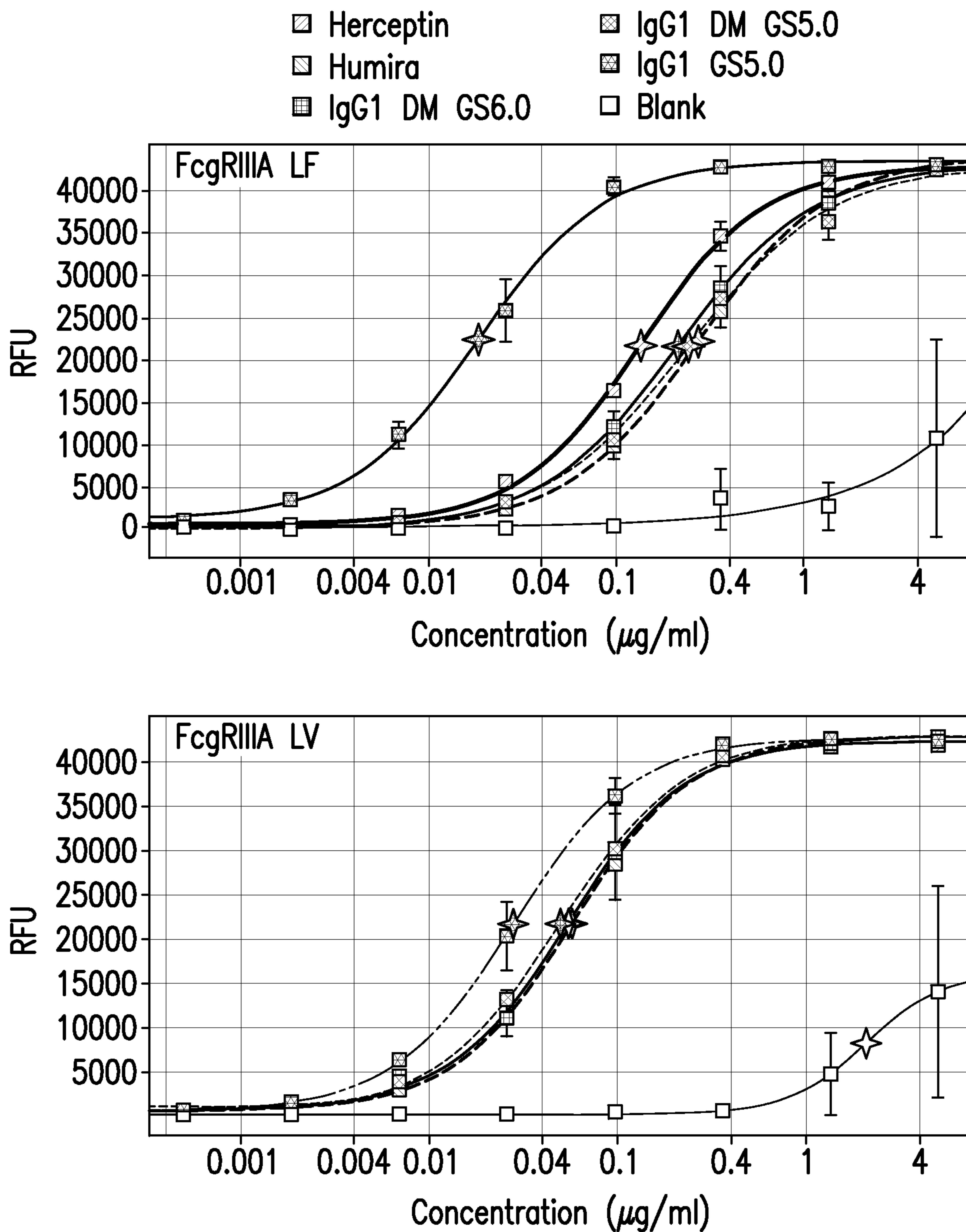


FIG.20-2

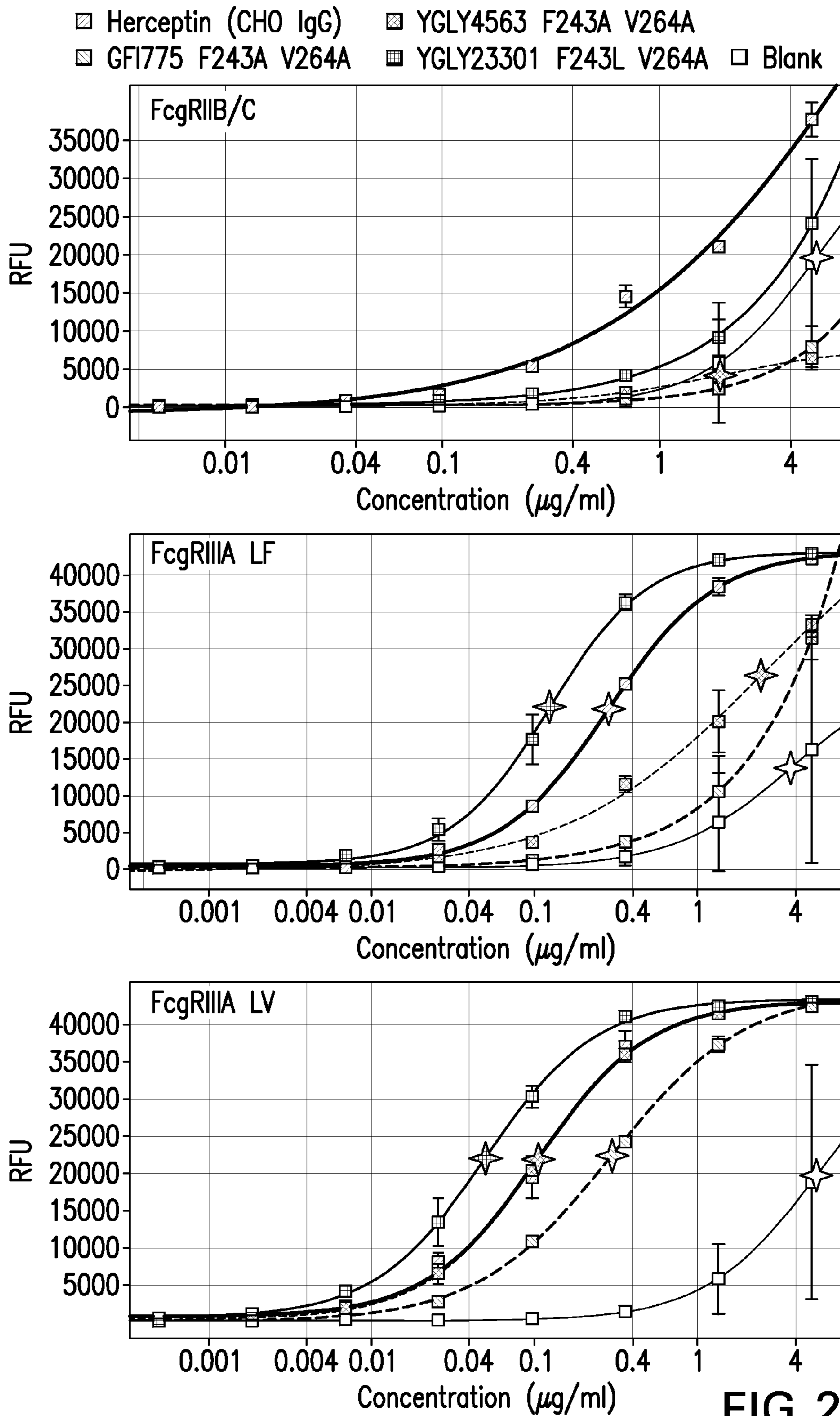


FIG.21A

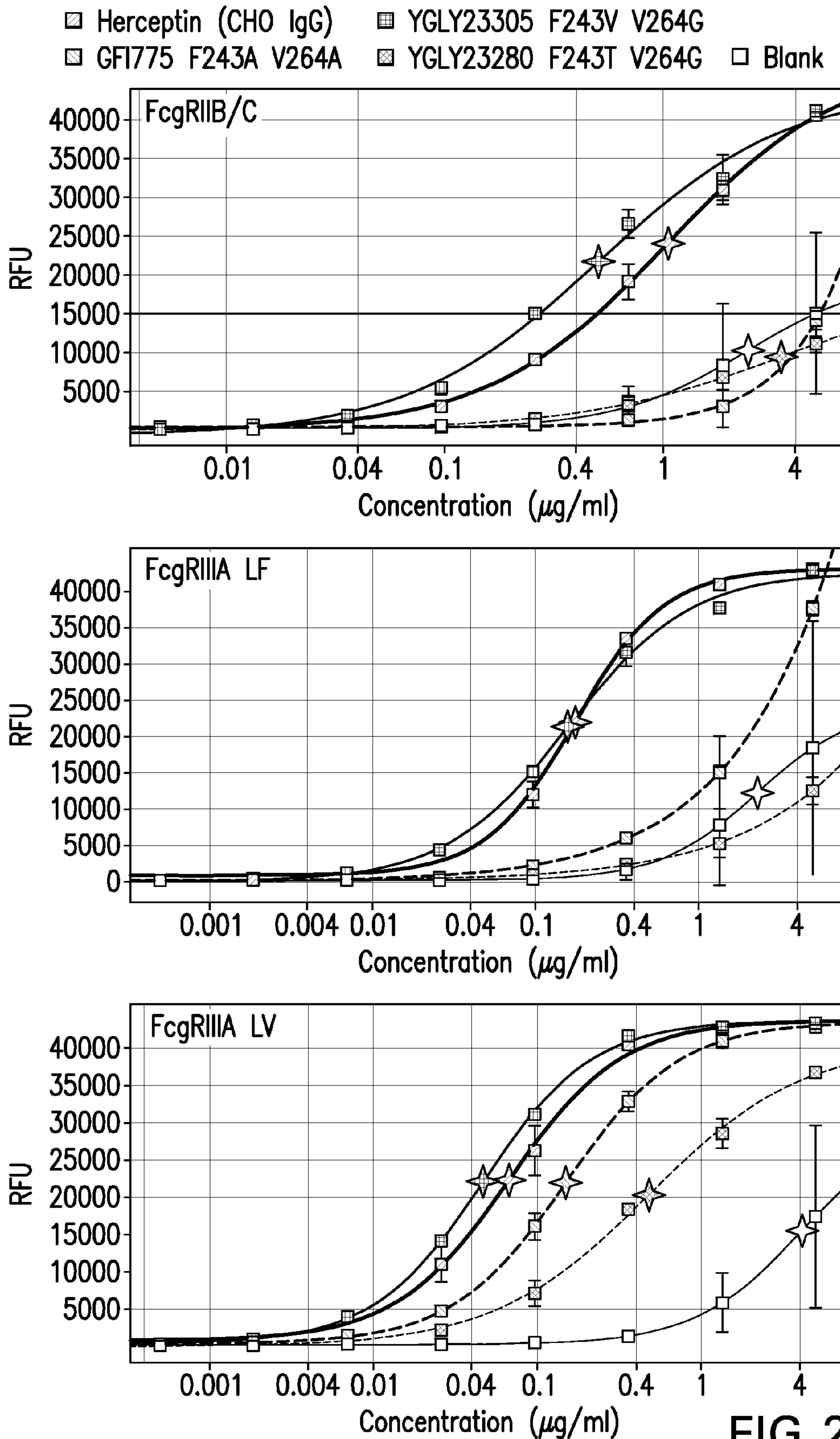


FIG. 21B

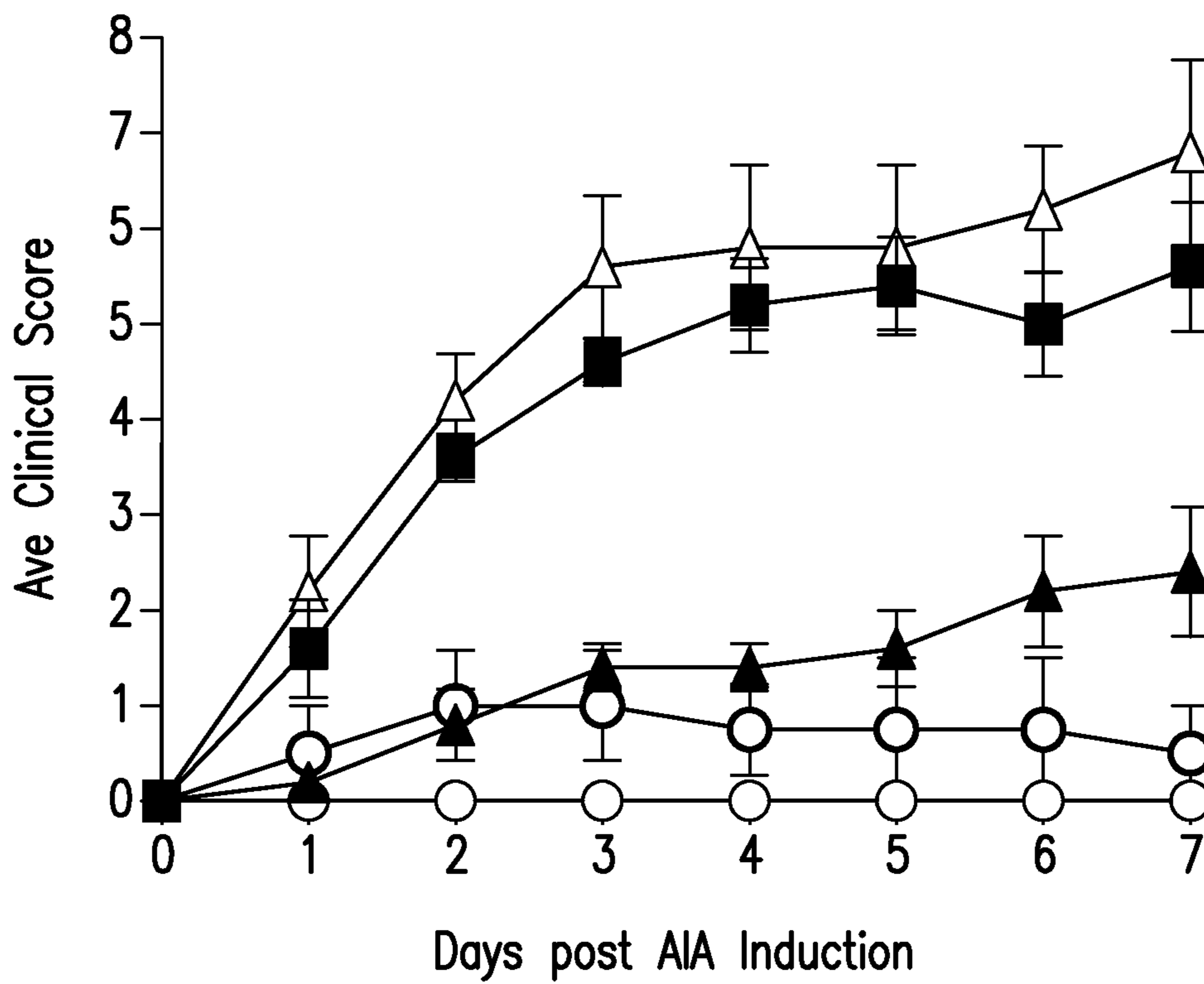
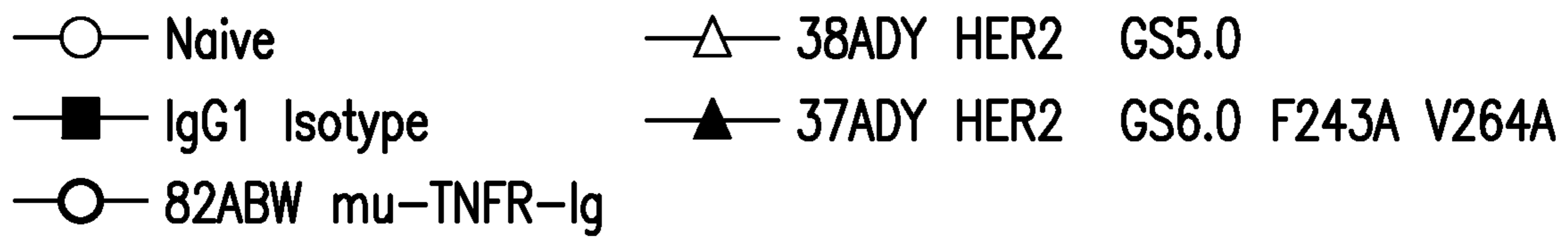


FIG.22

## METHOD FOR PREPARING ANTIBODIES HAVING IMPROVED PROPERTIES

### FIELD OF THE INVENTION

The present invention is directed to methods and compositions for the production of glycosylated proteins (glycoproteins) and, specifically, Fc-containing polypeptides which are useful as human or animal therapeutic agents.

The sequence listing of the present application is submitted electronically via EFS-Web as an ASCII formatted sequence listing with a file name "GFI\_MIS\_0003\_SeqListing.txt", creation date of Nov. 15, 2012 and a size of 40.5 KB. This sequence listing submitted via EFS-Web is part of the specification and is herein incorporated by reference in its entirety.

### BACKGROUND OF THE INVENTION

Monoclonal antibodies often achieve their therapeutic benefit through two binding events. First, the variable domain of the antibody binds a specific protein on a target cell, for example, CD20 on the surface of cancer cells. This is followed by recruitment of effector cells such as natural killer (NK) cells that bind to the constant region (Fc) of the antibody and destroy cells to which the antibody is bound. This process, known as antibody-dependent cell cytotoxicity (ADCC), depends on a specific N-glycosylation event at Asn 297 in the Fc domain of the heavy chain of IgG1s, Rothman et al., *Mol. Immunol.* 26: 1113-1123 (1989). Antibodies that lack this N-glycosylation structure still bind antigen but cannot mediate ADCC, apparently as a result of reduced affinity of the Fc domain of the antibody for the Fc Receptor FcγRIIIa on the surface of NK cells.

The presence of N-glycosylation not only plays a role in the effector function of an antibody, the particular composition of the N-linked oligosaccharide is also important for its end function. The lack of fucose or the presence of bisecting N-acetyl glucosamine has been positively correlated with the potency of the ADCC, Rothman (1989), Umana et al., *Nat. Biotech.* 17: 176-180 (1999), Shields et al., *J. Biol. Chem.* 277: 26733-26740 (2002), and Shinkawa et al., *J. Biol. Chem.* 278: 3466-3473 (2003). There is also evidence that sialylation in the Fc region is positively correlated with the anti-inflammatory properties of intravenous immunoglobulin (IVIg). See, e.g., Kaneko et al., *Science*, 313: 670-673, 2006; Nimmerjahn and Ravetch., *J. Exp. Med.*, 204: 11-15, 2007.

Given the utility of specific N-glycosylation in the function and potency of antibodies, a method for modifying the composition of N-linked oligosaccharides and modifying the effector function of antibodies would be desirable.

Yeast and other fungal hosts are important production platforms for the generation of recombinant proteins. Yeasts are eukaryotes and, therefore, share common evolutionary processes with higher eukaryotes, including many of the post-translational modifications that occur in the secretory pathway. Recent advances in glycoengineering have resulted in cell lines of the yeast strain *Pichia pastoris* with genetically modified glycosylation pathways that allow them to carry out a sequence of enzymatic reactions, which mimic the process of glycosylation in humans. See, for example, U.S. Pat. Nos. 7,029,872, 7,326,681 and 7,449,308 that describe methods for producing a recombinant glycoprotein in a lower eukaryote host cell that are substantially identical to their human counterparts. Human-like sialylated bi-antennary complex N-linked glycans like those produced in *Pichia pastoris* from the aforesaid methods have demonstrated utility for the production of therapeutic glycoproteins. Thus, a method for fur-

ther modifying or improving the production of antibodies in yeasts such as *Pichia pastoris* would be desirable.

### SUMMARY OF THE INVENTION

The invention relates to an Fc-containing polypeptide comprising mutations at amino acid positions 243 and 264 of the Fc region, wherein the mutations at positions 243 are selected from the group consisting of: F243A, F243G, F243S, F243T, F243V, F243L, F243I, F243D, F243Y, F243E, F243R, F243W and F243K and the mutations at position 264 are selected from the group consisting of: V264A, V264G, V264S, V264T, V264D, V264E, V264K, V264W, V264H, V264P, V264N, V264Q and V264L, wherein the numbering is according to the EU index as in Kabat. In another embodiment, the Fc-containing polypeptide comprises mutations F243A and V264A. In another embodiment, the Fc-containing polypeptide comprises mutations F243Y and V264G. In another embodiment, the Fc-containing polypeptide comprises mutations F243T and V264G. In another embodiment, the Fc-containing polypeptide comprises mutations F243L and V264A. In another embodiment, the Fc-containing polypeptide comprises mutations F243L and V264N. In another embodiment, the Fc-containing polypeptide comprises mutations F243V and V264G. In one embodiment, the Fc-containing polypeptide of the invention is an antibody or an antibody fragment. In one embodiment, the Fc-containing polypeptide of the invention is an antibody fragment comprising SEQ ID NO:18. In another embodiment, the Fc-containing polypeptide of the invention is an antibody fragment comprising SEQ ID NO:19. In another embodiment, the Fc-containing polypeptide of the invention is an antibody fragment consisting (or consisting essentially of) SEQ ID NO:18 or SEQ ID NO:19.

In one embodiment, the Fc-containing polypeptide is an antibody comprising the heavy chain amino acid sequence of SEQ ID NO:9 or a variant thereof, and the light chain amino acid sequence of SEQ ID NO:2 or a variant thereof. In one embodiment, the Fc-containing polypeptide is an antibody comprising the heavy chain amino acid sequence of SEQ ID NO:9 minus the last lysine (K) residue listed in SEQ ID NO:9.

In one embodiment, the Fc-containing polypeptide is an antibody comprising the heavy chain amino acid sequence of SEQ ID NO:12 or a variant thereof, and the light chain amino acid sequence of SEQ ID NO:11 or a variant thereof.

In one embodiment, the Fc-containing polypeptide is an antibody comprising the heavy chain amino acid sequence of SEQ ID NO:15 or a variant thereof, and the light chain amino acid sequence of SEQ ID NO:14 or a variant thereof.

In some embodiments, the Fc-containing polypeptides of the invention comprise N-glycans comprising sialic acid (including NANA, NGNA, and analogs and derivatives thereof). In one embodiment, the Fc-containing polypeptides of the invention comprise a mixture of  $\alpha$ -2,3 and  $\alpha$ -2,6 linked sialic acid. In another embodiment, the Fc-containing polypeptides of the invention comprise only  $\alpha$ -2,6 linked sialic acid. In one embodiment, the Fc-containing polypeptides of the invention comprise  $\alpha$ -2,6 linked sialic acid and comprise no detectable level of  $\alpha$ -2,3 linked sialic acid. In one embodiment, the sialic acid is N-acetylneuraminic acid (NANA) or N-glycolylneuraminic acid (NGNA) or a mixture thereof. In another embodiment, the sialic acid is an analog or derivative of NANA or NGNA with acetylation at position 9 on the sialic acid. In one embodiment, the N-glycans on the Fc-containing polypeptides of the invention comprise NANA and no NGNA.

The N-glycans on the Fc-containing polypeptides of the invention can optionally comprise fucose. In one embodiment, the N-glycans on the Fc-containing polypeptides will comprise a mixture of fucosylated and non-fucosylated N-glycans. In another embodiment, the N-glycans on the Fc-containing polypeptides lack fucose.

In one embodiment, the Fc-containing polypeptide of the invention has one or more of the following properties when compared to a parent Fc-containing polypeptide: (i) reduced effector function; (ii) increased anti-inflammatory properties; (iii) increased sialylation; (iv) increased bioavailability (absorption or exposure), and (v) reduced binding to FcγRI, FcγRIIa, FcγRIIb, FcγRIIIa (FcγRIIIa-V158 or FcγRIIIa-F158), and FcγRIIIb. In one embodiment, the Fc-containing polypeptide of the invention has at least a 7, 10, 15, 30, 50, 100, 500 or 1000 fold reduction in effector function compared to a parent Fc-containing polypeptide. In one embodiment, the effector function is ADCC. In another embodiment, the effector function is CDC.

In one embodiment, the Fc-containing polypeptide of the invention has reduced ADCC activity when compared to a parent Fc-containing polypeptide. In one embodiment, the Fc-containing polypeptide has at least a 7, 10, 15, 30, 50, 100, 500 or 1000 fold reduction in ADCC activity. In another embodiment, the Fc-containing polypeptide has at least a 100 fold reduction in ADCC activity. In another embodiment, the Fc-containing polypeptide has at least a 500 fold reduction in ADCC activity. In another embodiment, the Fc-containing polypeptide has at least a 1000 fold reduction in ADCC activity.

In another embodiment, the Fc-containing polypeptide of the invention has reduced CDC activity when compared to a parent Fc-containing polypeptide. In one embodiment, the Fc-containing polypeptide has at least 100 fold reduction in CDC activity.

In one embodiment, the Fc-containing polypeptide of the invention binds FcγRI, FcγRIIa, FcγRIIb, FcγRIIIa (FcγRIIIa-V158 or FcγRIIIa-F158), and FcγRIIIb with reduced affinity when compared to a parent Fc-containing polypeptide. In one embodiment, an Fc-containing polypeptide of the invention binds FcγRIIa with a reduced affinity of at least 50 fold when compared to a parent Fc-containing polypeptide. In one embodiment, the Fc-containing polypeptide of the invention binds FcγRIIb with a reduced affinity of at least 20 fold when compared to a parent Fc-containing polypeptide. In one embodiment, the Fc-containing polypeptide of the invention binds FcγRIIIa LF with a reduced affinity of at least 10 fold when compared to a parent Fc-containing polypeptide. In one embodiment, the Fc-containing polypeptide of the invention binds FcγRIIIa LV with a reduced affinity of at least 1, 2 or 10 fold when compared to a parent Fc-containing polypeptide. In one embodiment, the Fc-containing polypeptide of the invention binds FcγRIIb, FcγRIIIa LF and FcγRIIIa LV with a reduced affinity when compared to a parent Fc-containing polypeptide.

In one embodiment, the Fc-containing polypeptide of the invention has increased anti-inflammatory properties compared to a parent Fc-containing polypeptide.

In one embodiment, the Fc-containing polypeptide of the invention has increased bioavailability (absorption or exposure) when injected parenterally compared to a parent Fc-containing polypeptide. In one embodiment, the Fc-containing polypeptide of the invention has increased bioavailability (absorption or exposure) when injected subcutaneously compared to a parent Fc-containing polypeptide.

In a one embodiment, the parent Fc-containing polypeptide comprises a native Fc region. In another embodiment, the

parent Fc-containing polypeptide comprises a F243A mutation. In another embodiment, the parent Fc-containing polypeptide comprises a V264A mutation.

The invention also comprises a method for producing an Fc-containing polypeptide in a host cell comprising: (i) providing a host cell that has been genetically engineered to produce an Fc-containing polypeptide, wherein the host cell comprises a nucleic acid encoding mutations at amino acid positions 243 and 264 of the Fc region, wherein the mutations at positions 243 are selected from the group consisting of: F243A, F243G, F243S, F243T, F243V, F243L, F243I, F243D, F243Y, F243E, F243R, F243W and F243K and the mutations at position 264 are selected from the group consisting of: V264A, V264G, V264S, V264T, V264D, V264E, V264K, V264W, V264H, V264P, V264N, V264Q and V264L, wherein the numbering is according to the EU index as in Kabat; (ii) culturing the host cell under conditions which cause expression of the Fc-containing polypeptide; and (iii) isolating the Fc-containing polypeptide from the host cell. In one embodiment, the nucleic acid encodes the mutations F243A and V264A. In another embodiment, the nucleic acid encodes the mutations F243Y and V264G. In another embodiment, the nucleic acid encodes the mutations 243T and V264G. In another embodiment, the nucleic acid encodes the mutations F243L and V264A. In another embodiment, the nucleic acid encodes the mutations F243L and V264N. In another embodiment, the nucleic acid encodes the mutations F243V and V264G. In one embodiment, the Fc-containing polypeptide of the invention is an antibody or an antibody fragment. In one embodiment, the Fc-containing polypeptide is an antibody fragment comprising SEQ ID NO:18. In another embodiment, the Fc-containing polypeptide is an antibody fragment comprising SEQ ID NO:19. In another embodiment, the Fc-containing polypeptide is an antibody fragment consisting (or consisting essentially of) SEQ ID NO:18 or SEQ ID NO:19.

In one embodiment, the method for producing an Fc-containing polypeptide is carried out in a mammalian cell. In another embodiment, the method for producing an Fc-containing polypeptide is carried out in a plant cell. In another embodiment, the method for producing an Fc-containing polypeptide is carried out in bacteria. In another embodiment, the method for producing an Fc-containing polypeptide is carried out in an insect cell. In another embodiment, the method for producing an Fc-containing polypeptide is carried out in a lower eukaryotic cell. In another embodiment, the method for producing an Fc-containing polypeptide is carried out in a yeast cell. In one embodiment, the method for producing an Fc-containing polypeptide is carried out in *Pichia pastoris*.

In one embodiment, the Fc-containing polypeptide produced by the claimed method comprises N-glycans comprising sialic acid (including NANA, NGNA, and analogs and derivatives thereof). In one embodiment, the Fc-containing polypeptide produced by the claimed method has an N-glycan composition in which at least 40 mole %, 70 mole % or 90 mole % of the N-glycans on the Fc-containing polypeptide are sialylated (have a structure selected from  $SA_{(1-4)}Gal_{(1-4)}GlcNAc_{(2-4)}Man_3GlcNAc_2$  or  $SAGalGlcNAcMan_5GlcNAc_2$ ). In one embodiment, least 47 mole % of the N-glycans on the antibodies have the structure  $SA_2Gal_2GlcNAc_2Man_3GlcNAc_2$ . In another embodiment, least 47 mole % of the N-glycans on the antibodies have the structure  $NANA_2Gal_2GlcNAc_2Man_3GlcNAc_2$ . In another embodiment, least 66 mole % of the N-glycans on the antibodies have the structure  $SA_2Gal_2GlcNAc_2Man_3GlcNAc_2$ . In another embodiment,

least 66 mole % of the N-glycans on the antibodies have the structure  $\text{NANA}_2\text{Gal}_2\text{GlcNAc}_2\text{Man}_3\text{GlcNAc}_2$ . In one embodiment, the Fc-containing polypeptides produced by the claimed method comprise a mixture of  $\alpha$ -2,3 and  $\alpha$ -2,6 linked sialic acid. In another embodiment, the Fc-containing polypeptides comprise only  $\alpha$ -2,6 linked sialic acid. In one embodiment, the Fc-containing polypeptides of the invention comprise  $\alpha$ -2,6 linked sialic acid and comprise no detectable level of  $\alpha$ -2,3 linked sialic acid. In one embodiment, the sialic acid is N-acetylneuraminic acid (NANA) or N-glycolylneuraminic acid (NGNA) or a mixture thereof. In another embodiment, the sialic acid is an analog or derivative of NANA or NGNA with acetylation at position 9 on the sialic acid. In one embodiment, the N-glycans on the Fc-containing polypeptides produced by the claimed method comprise NANA and no NGNA.

The N-glycans on the Fc-containing polypeptides produced by the claimed method can optionally comprise fucose. In one embodiment, the N-glycans on the Fc-containing polypeptides produced by the claimed method comprise a mixture of fucosylated and non-fucosylated N-glycans. In one embodiment, the N-glycans on the Fc-containing polypeptides produced by the claimed method lack fucose.

In one embodiment, the Fc-containing polypeptide produced by the claimed method has an N-glycan composition in which the amount and percentage of total sialylated N-glycans is increased relative to a parent Fc-containing polypeptide.

In some embodiments, the Fc-containing polypeptide produced by the claimed method has one or more of the following properties when compared to a parent Fc-containing polypeptide: (i) reduced effector function; (ii) increased anti-inflammatory properties; (iii) increased sialylation; (iv) increased bioavailability (absorption or exposure), and (v) reduced binding to Fc $\gamma$ RI, Fc $\gamma$ RIIa, Fc $\gamma$ RIIb and Fc $\gamma$ RIIIa. In one embodiment, the Fc-containing polypeptide of the invention has at least a 7, 10, 15, 30, 50, 100, 500 or 1000 fold reduction in effector function relative to a parent Fc-containing polypeptide. In one embodiment, the effector function is ADCC. In another embodiment, the effector function is CDC.

In one embodiment, the Fc-containing polypeptide of the invention has reduced ADCC activity when compared to a parent Fc-containing polypeptide. In one embodiment, the Fc-containing polypeptide has at least a 7, 10, 15, 30, 50, 100, 500 or 1000 fold reduction in ADCC activity. In another embodiment, the Fc-containing polypeptide has at least a 100 fold reduction in ADCC activity. In another embodiment, the Fc-containing polypeptide has at least a 500 fold reduction in ADCC activity. In another embodiment, the Fc-containing polypeptide has at least a 1000 fold reduction in ADCC activity.

In another embodiment, the Fc-containing polypeptide produced by the claimed method has reduced CDC activity when compared to a parent Fc-containing polypeptide. In one embodiment, the Fc-containing polypeptide has at least 100 fold reduction in CDC activity.

In one embodiment, the Fc-containing polypeptide produced by the claimed method binds Fc $\gamma$ RI, Fc $\gamma$ RIIa, Fc $\gamma$ RIIb, Fc $\gamma$ RIIIa (Fc $\gamma$ RIIIa-V158 or Fc $\gamma$ RIIIa-F158), and Fc $\gamma$ RIIIb with reduced affinity when compared to a parent Fc-containing polypeptide. In one embodiment, the Fc-containing polypeptide of the invention binds Fc $\gamma$ RIIa with a reduced affinity of at least 50 fold when compared to a parent Fc-containing polypeptide. In one embodiment, the Fc-containing polypeptide of the invention binds Fc $\gamma$ RIIb with a reduced affinity of at least 20 fold when compared to a parent Fc-containing polypeptide. In one embodiment, the Fc-contain-

ing polypeptide of the invention binds Fc $\gamma$ RIIIa LF with a reduced affinity of at least 10 fold when compared to a parent Fc-containing polypeptide. In one embodiment, the Fc-containing polypeptide of the invention binds Fc $\gamma$ RIIIa LV with a reduced affinity of at least 1, 2 or 10 fold when compared to a parent Fc-containing polypeptide. In one embodiment, the Fc-containing polypeptide of the invention binds Fc $\gamma$ RIIb, Fc $\gamma$ RIIIa LF and Fc $\gamma$ RIIIa LV with a reduced affinity when compared to a parent Fc-containing polypeptide.

In one embodiment, the Fc-containing polypeptide produced by the claimed method has increased anti-inflammatory properties relative to a parent Fc-containing polypeptide.

In one embodiment, the Fc-containing polypeptide produced by the claimed method has increased bioavailability (absorption or exposure) when injected parenterally compared to a parent Fc-containing polypeptide. In one embodiment, the Fc-containing polypeptide produced by the claimed method has increased bioavailability (absorption or exposure) when injected subcutaneously compared to a parent Fc-containing polypeptide.

In a one embodiment, the parent Fc-containing polypeptide comprises a native Fc region. In another embodiment, the parent Fc-containing polypeptide comprises a F243A mutation. In another embodiment, the parent Fc-containing polypeptide comprises a V264A mutation.

The invention also comprises a method of reducing the effector function of an Fc-containing polypeptide, comprising introducing mutations at positions 243 and 264 of a parent Fc-containing polypeptide, wherein said Fc containing polypeptide has decreased effector function when compared to the parent Fc-containing polypeptide, wherein the numbering is according to the EU index as in Kabat. In a one embodiment, the Fc-containing polypeptide comprises mutations F243A and V264A. In another embodiment, the nucleic acid encodes the mutations F243Y and V264G. In another embodiment, the nucleic acid encodes the mutations 243T and V264G. In another embodiment, the nucleic acid encodes the mutations F243L and V264A. In another embodiment, the nucleic acid encodes the mutations F243L and V264N. In another embodiment, the nucleic acid encodes the mutations F243V and V264G. In one embodiment, the effector function is ADCC. In another embodiment, the effector function is CDC. In one embodiment, the Fc-containing polypeptide of the invention is an antibody or an antibody fragment. In one embodiment, the Fc-containing polypeptide is an antibody fragment comprising SEQ ID NO:18. In another embodiment, the Fc-containing polypeptide is an antibody fragment comprising SEQ ID NO:19. In another embodiment, the Fc-containing polypeptide is an antibody fragment consisting (or consisting essentially of) SEQ ID NO:18 or SEQ ID NO:19. In a one embodiment, the parent Fc-containing polypeptide comprises a native Fc region. In another embodiment, the parent Fc-containing polypeptide comprises a F243A mutation. In another embodiment, the parent Fc-containing polypeptide comprises a V264A mutation.

The invention also comprises a method of increasing the anti-inflammatory properties of an Fc-containing polypeptide, comprising introducing mutations at positions 243 and 264 of a parent Fc-containing polypeptide, wherein the numbering is according to the EU index as in Kabat, wherein said Fc containing polypeptide has increased anti-inflammatory activity when compared to a parent Fc-containing polypeptide. In a one embodiment, the Fc-containing polypeptide comprises mutations F243A and V264A. In another embodiment, the Fc-containing polypeptide comprises mutations F243Y and V264G. In another embodiment, the Fc-containing polypeptide comprises mutations 243T and V264G. In

another embodiment, the Fc-containing polypeptide comprises mutations F243L and V264A. In another embodiment, the Fc-containing polypeptide comprises mutations F243L and V264N. In another embodiment, the Fc-containing polypeptide comprises mutations F243V and V264G. In one embodiment, the Fc-containing polypeptide of the invention is an antibody or an antibody fragment. In one embodiment, the Fc-containing polypeptide is an antibody or antigen binding fragment thereof that binds to an antigen selected from the group consisting of: TNF- $\alpha$ , IL-1, IL-2, IL-4, IL-5, IL-6, IL-8, IL-9, IL-10, IL-12, IL-15, IL-17, IL-18, IL-20, IL-21, IL-22, IL-23, IL-23R, IL-25, IL-27, IL-33, CD2, CD4, CD11A, CD14, CD18, CD19, CD23, CD25, CD40, CD40L, CD20, CD52, CD64, CD80, CD147, CD200, CD200R, TSLP, TSLPR, PD-1, PDL1, CTLA4, VLA-4, VEGF, PCSK9,  $\alpha$ 4 $\beta$ 7-integrin, E-selectin, Fact II, ICAM-3, beta2-integrin, IFN $\gamma$ , C5, CBL, LCAT, CR3, MDL-1, GITR, ADDL, CGRP, TRKA, IGFI1R, RANKL, GTC, or the receptor for any of the above mentioned molecules. In a one embodiment, the Fc-containing polypeptide will bind to TNF- $\alpha$ . In another embodiment, the Fc-containing polypeptide will bind to Her2. In another embodiment, the Fc-containing polypeptide will bind to PCSK9. In one embodiment, the Fc-containing polypeptide of the invention is an antibody fragment comprising SEQ ID NO:18. In another embodiment, the Fc-containing polypeptide of the invention is an antibody fragment comprising SEQ ID NO:19. In another embodiment, the Fc-containing polypeptide of the invention is an antibody fragment consisting (or consisting essentially of) SEQ ID NO:18 or SEQ ID NO:19. In one embodiment, the parent Fc-containing polypeptide comprises a native Fc region. In another embodiment, the parent Fc-containing polypeptide comprises a F243A mutation. In another embodiment, the parent Fc-containing polypeptide comprises a V264A mutation.

The invention also comprises a method of increasing the anti-inflammatory properties of an Fc-containing polypeptide comprising: selecting a parent Fc-containing polypeptide that is useful in treating inflammation (for example, an antibody or immunoadhesin that binds to an antigen that is involved in inflammation) and introducing mutations at positions 243 and 264 of the Fc-region, wherein the numbering is according to the EU index as in Kabat, wherein the Fc-containing polypeptide has increased anti-inflammatory activity when compared to the parent Fc-containing polypeptide. In a one embodiment, the Fc-containing polypeptide comprises mutations F243A and V264A. In another embodiment, the Fc-containing polypeptide comprises mutations F243Y and V264G. In another embodiment, the Fc-containing polypeptide comprises mutations 243T and V264G. In another embodiment, the Fc-containing polypeptide comprises mutations F243L and V264A. In another embodiment, the Fc-containing polypeptide comprises mutations F243L and V264N. In another embodiment, the Fc-containing polypeptide comprises mutations F243V and V264G. In one embodiment, the Fc-containing polypeptide of the invention is an antibody or an antibody fragment. In one embodiment, the Fc-containing polypeptide is an antibody or antigen binding fragment thereof that binds to an antigen selected from the group consisting of: TNF- $\alpha$ , IL-1, IL-2, IL-4, IL-5, IL-6, IL-8, IL-9, IL-10, IL-12, IL-15, IL-17, IL-18, IL-20, IL-21, IL-22, IL-23, IL-23R, IL-25, IL-27, IL-33, CD2, CD4, CD11A, CD14, CD18, CD19, CD23, CD25, CD40, CD40L, CD20, CD52, CD64, CD80, CD147, CD200, CD200R, TSLP, TSLPR, PD-1, PDL1, CTLA4, VLA-4, VEGF, PCSK9,  $\alpha$ 4 $\beta$ 7-integrin, E-selectin, Fact II, ICAM-3, beta2-integrin, IFN $\gamma$ , C5, CBL, LCAT, CR3, MDL-1, GITR,

ADDL, CGRP, TRKA, IGFI1R, RANKL, GTC, or the receptor for any of the above mentioned molecules. In a one embodiment, the Fc-containing polypeptide will bind to TNF- $\alpha$ . In another one embodiment, the Fc-containing polypeptide will bind to Her2. In another one embodiment, the Fc-containing polypeptide will bind to PCSK9. In one embodiment, the Fc-containing polypeptide is an antibody fragment comprising SEQ ID NO:18. In another embodiment, the Fc-containing polypeptide is an antibody fragment comprising SEQ ID NO:19. In another embodiment, the Fc-containing polypeptide is an antibody fragment consisting (or consisting essentially of) SEQ ID NO:18 or SEQ ID NO:19. In one embodiment, the parent Fc-containing polypeptide comprises a native Fc region. In another embodiment, the parent Fc-containing polypeptide comprises a F243A mutation. In another embodiment, the parent Fc-containing polypeptide comprises a V264A mutation.

The invention also comprises a method of treating an inflammatory condition in a subject in need thereof comprising: administering to the subject a therapeutically effective amount of an Fc-containing polypeptide comprising mutations at positions 243 and 264, wherein the numbering is according to the EU index as in Kabat. In one embodiment, the Fc-containing polypeptide decreases the expression of a gene selected from the group consisting of: IL-1 $\beta$ , IL-6, RANKL, TRAP, ATP6v0d2, MDL-1, DAP12, CD11b, TIMP-1, MMP9, CTSK, PU-1, MCP1, MIP1 $\alpha$ , Cxcl1-Groa, CXcl2-Grob, CD18, TNF, Fc $\gamma$ RI, Fc $\gamma$ RIIb, Fc $\gamma$ RIII and Fc $\gamma$ RIV. In a one embodiment, the Fc-containing polypeptide comprises mutations F243A and V264A. In another embodiment, the nucleic acid encodes the mutations F243Y and V264G. In another embodiment, the nucleic acid encodes the mutations 243T and V264G. In another embodiment, the nucleic acid encodes the mutations F243L and V264A. In another embodiment, the nucleic acid encodes the mutations F243L and V264N. In another embodiment, the nucleic acid encodes the mutations F243V and V264G. In one embodiment, the Fc-containing polypeptide is administered parenterally. In one embodiment, the Fc-containing polypeptide is administered subcutaneously. In one embodiment, the Fc-containing polypeptide is an antibody or antigen binding fragment thereof. In one embodiment, the Fc-containing polypeptide is an antibody or antigen binding fragment thereof that is useful in treating an inflammatory condition. In one embodiment, the antibody or antigen binding fragment thereof binds to an antigen selected from the group consisting of: TNF- $\alpha$ , IL-1, IL-2, IL-4, IL-5, IL-6, IL-8, IL-9, IL-10, IL-12, IL-15, IL-17, IL-18, IL-20, IL-21, IL-22, IL-23, IL-23R, IL-25, IL-27, IL-33, CD2, CD4, CD11A, CD14, CD18, CD19, CD23, CD25, CD40, CD40L, CD20, CD52, CD64, CD80, CD147, CD200, CD200R, TSLP, TSLPR, PD-1, PDL1, CTLA4, VLA-4, VEGF, PCSK9,  $\alpha$ 4 $\beta$ 7-integrin, E-selectin, Fact II, ICAM-3, beta2-integrin, IFN $\gamma$ , C5, CBL, LCAT, CR3, MDL-1, GITR, ADDL, CGRP, TRKA, IGFI1R, RANKL, GTC, or the receptor for any of the above mentioned molecules. In one embodiment, the Fc-containing polypeptide will bind to TNF- $\alpha$ . In another embodiment, the Fc-containing polypeptide will bind to Her2. In another embodiment, the Fc-containing polypeptide will bind to PCSK9. In one embodiment, the Fc-containing polypeptide is an antibody fragment comprising SEQ ID NO:18. In another embodiment, the Fc-containing polypeptide is an antibody fragment comprising SEQ ID NO:19. In another embodiment, the Fc-containing polypeptide is an antibody fragment consisting (or consisting essentially of) SEQ ID NO:18 or SEQ ID NO:19



Another invention disclosed herein relates to a pharmaceutical composition comprising an Fc-containing polypeptide, wherein at least 70% of the N-glycans on the Fc-containing polypeptide comprise an oligosaccharide structure selected from the group consisting of SA<sub>(1-4)</sub>Gal<sub>(1-4)</sub>GlcNAc<sub>(2-4)</sub> Man<sub>3</sub>GlcNAc<sub>2</sub> and SAGalGlcNAcMan<sub>5</sub>GlcNAc<sub>2</sub>, wherein the Fc-containing polypeptide comprises mutations at amino acid positions 243 and 264 of the Fc region, wherein the numbering is according to the EU index as in Kabat. In one embodiment, the mutations are F243A and V264A. In another embodiment, the mutations are F243Y and V264G. In another embodiment, the nucleic acid encodes the mutations 243T and V264G. In another embodiment, the mutations are F243L and V264A. In another embodiment, the mutations are F243L and V264N. In another embodiment, the mutations are F243V and V264G. In one embodiment, at least 47 mole % of the N-glycans have the structure SA<sub>2</sub>Gal<sub>2</sub>GlcNAc<sub>2</sub>Man<sub>3</sub>GlcNAc<sub>2</sub>. In another embodiment, at least 47 mole % of the N-glycans have the structure NANA<sub>2</sub>Gal<sub>2</sub>GlcNAc<sub>2</sub>Man<sub>3</sub>GlcNAc<sub>2</sub>. In one embodiment, the sialylated N-glycans comprise a mixture of  $\alpha$ -2,3 and  $\alpha$ -2,6 linked sialic acid. In another embodiment, the sialylated N-glycans comprise only  $\alpha$ -2,6 linked sialic acid. In another embodiment, the sialylated N-glycans comprise  $\alpha$ -2,6 linked sialic acid and comprise no detectable level of  $\alpha$ -2,3 linked sialic acid. In one embodiment, the sialic acid is N-acetylneuraminic acid (NANA) or N-glycolylneuraminic acid (NGNA) or a mixture thereof. In another embodiment, the sialic acid is an analog or derivative of NANA or NGNA with acetylation at position 9 on the sialic acid. In one embodiment, the N-glycans on the Fc-containing polypeptides comprise NANA and no NGNA. In one embodiment, the Fc-containing polypeptide is an antibody fragment comprising SEQ ID NO:18. In another embodiment, the Fc-containing polypeptide is an antibody fragment comprising SEQ ID NO:19. In another embodiment, the Fc-containing polypeptide is an antibody fragment consisting (or consisting essentially of) SEQ ID NO:18 or SEQ ID NO:19

Another invention disclosed herein relates to a pharmaceutical composition comprising an Fc-containing polypeptide, wherein at least 70% of the N-glycans on the Fc-containing polypeptide comprise an oligosaccharide structure selected from the group consisting of SA<sub>(1-4)</sub>Gal<sub>(1-4)</sub>GlcNAc<sub>(2-4)</sub> Man<sub>3</sub>GlcNAc<sub>2</sub> and SAGalGlcNAcMan<sub>5</sub>GlcNAc<sub>2</sub>, wherein the sialic acid residues are attached exclusively via an  $\alpha$ -2,6 linkage, wherein the N-glycans lack fucose, and wherein the Fc-containing polypeptide comprises mutations at amino acid positions 243 and 264 of the Fc region, wherein the numbering is according to the EU index as in Kabat. In one embodiment, the mutations are F243A and V264A. In another embodiment, the mutations are F243Y and V264G. In another embodiment, the nucleic acid encodes the mutations 243T and V264G. In another embodiment, the mutations are F243L and V264A. In another embodiment, the mutations are F243L and V264N. In another embodiment, the mutations are F243V and V264G. In one embodiment, at least 47 mole % of the N-glycans have the structure SA<sub>2</sub>Gal<sub>2</sub>GlcNAc<sub>2</sub>Man<sub>3</sub>GlcNAc<sub>2</sub>. In another embodiment, at least 47 mole % of the N-glycans have the structure NANA<sub>2</sub>Gal<sub>2</sub>GlcNAc<sub>2</sub>Man<sub>3</sub>GlcNAc<sub>2</sub>. In one embodiment, the sialylated N-glycans comprise a mixture of  $\alpha$ -2,3 and  $\alpha$ -2,6 linked sialic acid. In another embodiment, the sialylated N-glycans comprise only  $\alpha$ -2,6 linked sialic acid. In another embodiment, the sialylated N-glycans comprise  $\alpha$ -2,6 linked sialic acid and comprise no detectable level of  $\alpha$ -2,3 linked sialic acid. In one embodiment, the sialic acid is N-acetylneuraminic acid (NANA) or N-glycolylneuraminic

acid (NGNA) or a mixture thereof. In another embodiment, the sialic acid is an analog or derivative of NANA or NGNA with acetylation at position 9 on the sialic acid. In one embodiment, the N-glycans on the Fc-containing polypeptides comprise NANA and no NGNA. In one embodiment, the Fc-containing polypeptide is an antibody fragment comprising SEQ ID NO:18. In another embodiment, the Fc-containing polypeptide is an antibody fragment comprising SEQ ID NO:19. In another embodiment, the Fc-containing polypeptide is an antibody fragment consisting (or consisting essentially of) SEQ ID NO:18 or SEQ ID NO:19

The invention also comprises an Fc-containing polypeptide comprising a heavy chain and a light chain, wherein the heavy chain comprises the amino acid sequence of SEQ ID NO:9 or a variant thereof and the light chain comprises the amino acid sequence of SEQ ID NO:2 or a variant thereof, wherein the variant comprises one or more of the following properties when compared to an antibody comprising the heavy chain amino acid sequence of SEQ ID NO:1 and the light chain amino acid sequence of SEQ ID NO:2: reduced effector function, increased anti-inflammatory properties; increased sialylation; increased bioavailability (absorption or exposure) when administered parenterally, and reduced binding to Fc $\gamma$ RI, Fc $\gamma$ RIIa, Fc $\gamma$ RIIb and Fc $\gamma$ RIIIa. The invention also comprises an Fc-containing polypeptide comprising a heavy chain and a light chain, wherein the heavy chain comprises the amino acid sequence of SEQ ID NO:12 or a variant thereof and the light chain comprises the amino acid sequence of SEQ ID NO:11 or a variant thereof, wherein the variant comprises one or more of the following properties when compared to an antibody comprising the heavy chain amino acid sequence of SEQ ID NO:10 and the light chain amino acid sequence of SEQ ID NO:11: reduced effector function, increased anti-inflammatory properties, increased sialylation, increased bioavailability (absorption or exposure) when administered parenterally, and reduced binding to Fc $\gamma$ RI, Fc $\gamma$ RIIa, Fc $\gamma$ RIIb, Fc $\gamma$ RIIIa and Fc $\gamma$ RIIIb. The invention also comprises an Fc-containing polypeptide comprising a heavy chain and a light chain, wherein the heavy chain comprises the amino acid sequence of SEQ ID NO:15 or a variant thereof, and the light chain comprises the amino acid sequence of SEQ ID NO:14 or a variant thereof, wherein the variant comprises one or more of the following properties when compared to an antibody comprising the heavy chain amino acid sequence of SEQ ID NO:13 and the light chain amino acid sequence of SEQ ID NO:14: reduced effector function, increased anti-inflammatory properties, increased sialylation, increased bioavailability (absorption or exposure) when administered parenterally, and reduced binding to Fc $\gamma$ RI, Fc $\gamma$ RIIa, Fc $\gamma$ RIIb, Fc $\gamma$ RIIIa and Fc $\gamma$ RIIIb. In one embodiment, the variant comprises up to 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more conservative or non conservative amino acid substitutions. In one embodiment, the variant comprises at least 75%, 80%, 85%, 90%, 95%, 98% or 99% sequence identity with the claimed sequence

The invention also comprises a method of increasing the anti-inflammatory properties of an Fc-containing polypeptide, comprising introducing a mutation at position 243 or a mutation at position 264 of a parent Fc-containing polypeptide, wherein the numbering is according to the EU index as in Kabat, wherein said Fc containing polypeptide has increased anti-inflammatory function when compared to the parent Fc-containing polypeptide. The invention also comprises a method of increasing the anti-inflammatory properties of an Fc-containing polypeptide comprising: selecting a parent Fc-containing polypeptide that is useful in treating inflammation (for example, an antibody or immunoadhesin

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that binds to an antigen that is involved in inflammation) and introducing a mutation at position 243 or a mutation at position 264 of a parent Fc-containing polypeptide, wherein the numbering is according to the EU index as in Kabat, wherein the Fc-containing polypeptide has increased anti-inflammatory properties when compared to the parent Fc-containing polypeptide. In one embodiment, the Fc-containing polypeptide comprises mutation F243A. In another embodiment, the Fc-containing polypeptide comprises mutation V264A. In one embodiment, the parent Fc-containing polypeptide comprises a native Fc region.

The invention also comprises a method of treating an inflammatory condition in a subject in need thereof comprising: administering to the subject a therapeutically effective amount of an Fc-containing polypeptide a mutation at position 243 or a mutation at position 264 of a parent Fc-containing polypeptide, wherein the numbering is according to the EU index as in Kabat. In one embodiment, the Fc-containing polypeptide is administered parenterally. In one embodiment, the Fc-containing polypeptide is administered subcutaneously. In one embodiment, the Fc-containing polypeptide comprises mutation F243A. In another embodiment, the Fc-containing polypeptide comprises mutation V264A. In one embodiment, the parent Fc-containing polypeptide comprises a native Fc region.

In any of the above embodiment, an increase in anti-inflammatory activity can be detected using any method known in the art. In one embodiment, an increase in anti-inflammatory activity is detected by measuring a decrease in the expression of a gene selected from the group consisting of: IL-1 $\beta$ , IL-6, RANKL, TRAP, ATP6v0d2, MDL-1, DAP12, CD11b, TIMP-1, MMP9, CTSK, PU-1, MCP1, MIP1 $\alpha$ , Cxcl1-Groa, CXcl2-Grob, CD18, TNF, Fc $\gamma$ RI, Fc $\gamma$ RIIb, Fc $\gamma$ RIII and Fc $\gamma$ RIV.

## BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a graphic representation of pGLY3483, the F243A and V264A double mutein expression plasmid. Both heavy and light chains were under the control of a methanol inducible promoter, AOX1. The PpTrp2 gene was the locus applied to integrate the entire cassette. With the exception of the mutations on the heavy chain, expression plasmid structure was the same for the wild type (parent), single F243A mutein, single V264A mutein, and the double mutein expression plasmids.

FIG. 2 is a representation of the gels from an SDS-PAGE analysis characterizing the non-reduced (NR) and reduced (R) antibodies produced by the materials and methods herein. Lane 1 contains an anti-Her2 monoclonal antibody, Her2; Lane 2 contains a single Fc mutein, F243A; Lane 3 contains a single Fc mutein, V264A; and Lane 4 contains a double Fc mutein, F243A/V264A.

FIG. 3 illustrates antigen affinity for various antibodies produced by the materials and methods herein as determined by a cell based assay using an SK-BR3 cell line, a Her2-overexpressing human breast cancer line. ■—Her2; ◆—F243A/V264A GS6.0 glycosylation; ▲—F243A with GS6.0 glycosylation; ▼—V264A with GS6.0 glycosylation; ◇—Control IgG; x—*Pichia pastoris* Her2 produced with GFI 5.0 glycosylation.

FIG. 4 illustrates a MALDI-TOF MS analysis of N-glycans of a *Pichia pastoris* Her2 antibody produced in GFI 5.0 strain YDX477. The peaks are Man<sub>5</sub>GlcNAc<sub>2</sub>, 1261.24 (GS2.0), GlcNAc<sub>2</sub>Man<sub>3</sub>GlcNAc<sub>2</sub>, 1343.50 (G0) (predominant), GalGlcNAc<sub>2</sub>Man<sub>3</sub>GlcNAc<sub>2</sub>, 1505.97 (G1), and Gal<sub>2</sub>GlcNAc<sub>2</sub>Man<sub>3</sub>GlcNAc<sub>2</sub>, 1668.47 (G2).

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FIG. 5 illustrates a MALDI-TOF MS analysis of N-glycans of a single Fc mutein, F243A, antibody produced in GFI 5.0 strain YDX551. The peaks are Man<sub>5</sub>GlcNAc<sub>2</sub>, 1261.05, GlcNAc<sub>2</sub>Man<sub>3</sub>GlcNAc<sub>2</sub>, 1343.71, GalGlcNAc<sub>2</sub>Man<sub>3</sub>GlcNAc<sub>2</sub>, 1506.62, and Gal<sub>2</sub>GlcNAc<sub>2</sub>Man<sub>3</sub>GlcNAc<sub>2</sub>, 1668.97 (predominant).

FIG. 6 illustrates a MALDI-TOF MS analysis of N-glycans of a single Fc mutein, V264A, antibody produced in GFI 5.0 strain YDX551. The peaks are Man<sub>5</sub>GlcNAc<sub>2</sub>, 1261.98, GalGlcNAc<sub>2</sub>Man<sub>3</sub>GlcNAc<sub>2</sub>, 1505.45, and Gal<sub>2</sub>GlcNAc<sub>2</sub>Man<sub>3</sub>GlcNAc<sub>2</sub>, 1668.85 (predominant).

FIG. 7 illustrates a MALDI-TOF MS analysis of N-glycans of a double Fc mutein, F243A/V264A, antibody produced in GFI 5.0 strain YDX557. The major peak corresponds to Gal<sub>2</sub>GlcNAc<sub>2</sub>Man<sub>3</sub>GlcNAc<sub>2</sub>, 1668.39.

FIG. 8 illustrates a MALDI-TOF MS analysis of N-glycans of a double Fc mutein, F243A/V264A, antibody produced in GFI 6.0 strain YGLY4563. The double peaks at 2224.28 and 2245.83 (predominant) correspond to NANAGal<sub>2</sub>GlcNAc<sub>2</sub>Man<sub>3</sub>GlcNAc<sub>2</sub> and NANA<sub>2</sub>Gal<sub>2</sub>GlcNAc<sub>2</sub>Man<sub>3</sub>GlcNAc<sub>2</sub>, respectively.

FIGS. 9A-9D are graphic representations of the Fc $\gamma$ R binding for various antibodies produced by the materials and methods described in Example 11: Fc $\gamma$ RIIIaLF (FIG. 9A); Fc $\gamma$ RI (FIG. 9B); Fc $\gamma$ RIIb/c (FIG. 9C); and Fc $\gamma$ RIIIaLV (FIG. 9D). For FIGS. 9A-9D: ■—Her2; ▲—F243A produced in GFI 6.0; ▼—V264A produced in GFI 6.0; ◆—F243A/V264A produced in GFI 6.0; ●—F243A/V264A produced in GFI 6.0 and treated with PNGase.

FIG. 10 is a graphic representation of the C1q binding for various antibodies produced by the materials and methods described in Example 12: ◇—anti-CD20 antibody, a positive control; ■—Her2; ▲—F243A produced with GS6.0 glycosylation; ▼—V264A produced with GS6.0 glycosylation; ◆—F243A/V264A produced with GS6.0 glycosylation; ●—F243A/V264A with GS6.0 glycosylation and PNGase.

FIG. 11 is a graphic representation of the ADCC response for the various antibodies produced by the materials and methods described in Example 13: ■—Her2; ▲—F243A produced with GS6.0 glycosylation; ▼—V264A produced with GS6.0 glycosylation; ◆—F243A/V264A produced with GS6.0 glycosylation; ◇—NK cell killing without antibody; X—Her2 produced in GFI2.0.

FIG. 12 is a graphic representation of serum monoclonal antibody concentration over time for mice injected with: ■—Her2; X—Her2 produced in GFI 5.0; ◆—F243A/V264A produced in GFI 6.0 as described in Example 14.

FIGS. 13A-13E are graphic representations of the Fc $\gamma$ R binding for various antibodies described in Example 15.

FIG. 14 is a graphic representation of the ADCC response for the various antibodies produced by the materials and methods described in Example 16. The results in FIGS. 14A and 14B were from experiments using heterozygous F/V effector cells. The results in FIGS. 14C and 14D were from experiments using F/F effector cells. The results in FIG. 14E were from an experiment using V/V effector cells.

FIG. 15 is a graphic representation of the ADCC activity of the anti-Her2 Fc double mutant compared to the assumed additive reference curve of each of the single mutants as described in Example 17.

FIG. 16 is a graphic representation of the ADCC response for the various antibodies produced by the materials and methods described in Example 18.

FIG. 17 is a graphic representation of the CDC response for the various antibodies produced by the materials and methods described in Example 19.

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FIG. 18 is a graphic representation of the effect of the Fc muteins of the invention in an AIA model described in Example 20.

FIG. 19 is a graphic representation of the effect of the Fc muteins of the invention in an AIA model described in Example 21.

FIG. 20 is a graphic representation of the Fc $\gamma$ R binding for the anti-TNF $\alpha$  antibodies described in Example 22.

FIG. 21 is a graphic representation of the Fc $\gamma$ R binding for the anti-TNF $\alpha$  antibodies described in Example 23.

FIG. 22 is a graphic representation of the effect of the Fc muteins of the invention in an AIA model described in Example 24.

## DETAILED DESCRIPTION OF THE INVENTION

## Definitions

The term “G0” when used herein refers to a complex bi-antennary oligosaccharide without galactose or fucose, GlcNAc<sub>2</sub>Man<sub>3</sub>GlcNAc<sub>2</sub>.

The term “G1” when used herein refers to a complex bi-antennary oligosaccharide without fucose and containing one galactosyl residue, GalGlcNAc<sub>2</sub>Man<sub>3</sub>GlcNAc<sub>2</sub>.

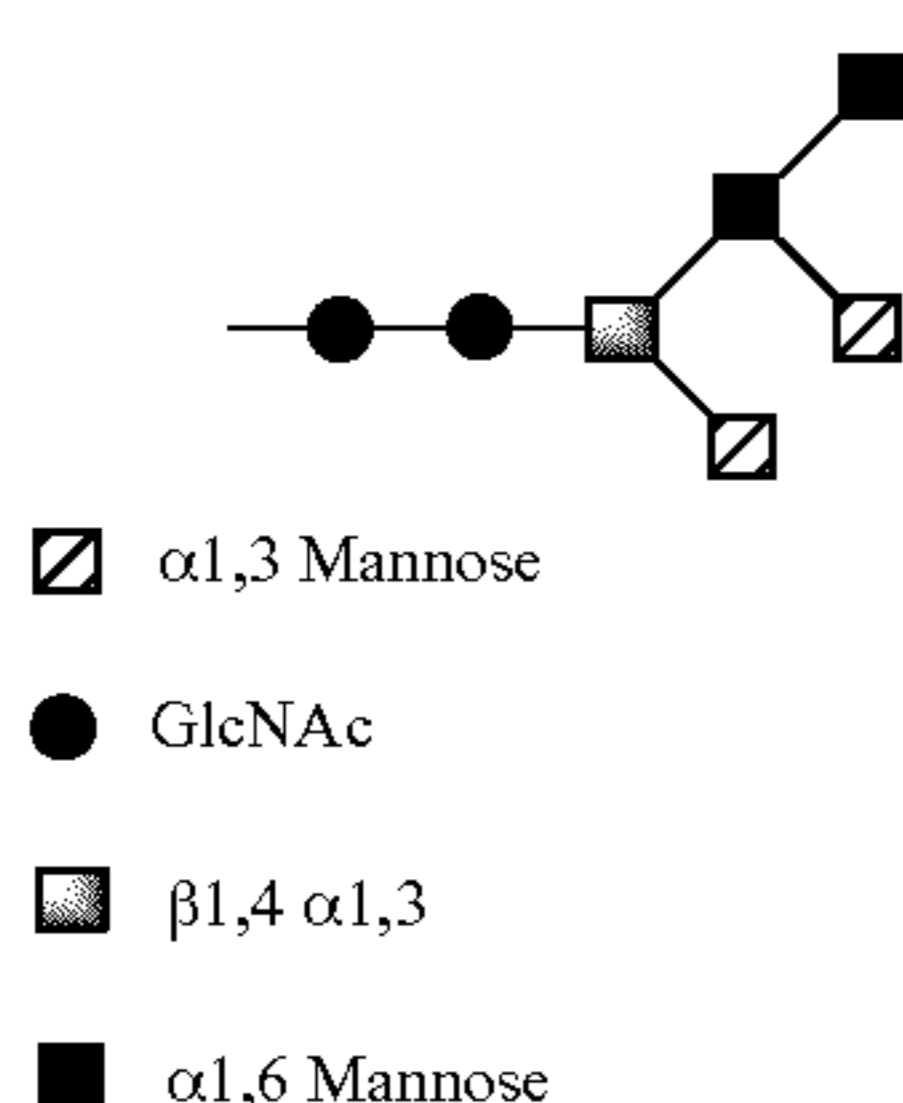
The term “G2” when used herein refers to a complex bi-antennary oligosaccharide without fucose and containing two galactosyl residues, Gal<sub>2</sub>GlcNAc<sub>2</sub>Man<sub>3</sub>GlcNAc<sub>2</sub>.

The term “G0F” when used herein refers to a complex bi-antennary oligosaccharide containing a core fucose and without galactose, GlcNAc<sub>2</sub>Man<sub>3</sub>GlcNAc<sub>2</sub>F.

The term “G1F” when used herein refers to a complex bi-antennary oligosaccharide containing a core fucose and one galactosyl residue, GalGlcNAc<sub>2</sub>Man<sub>3</sub>GlcNAc<sub>2</sub>F.

The term “G2F” when used herein refers to a complex bi-antennary oligosaccharide containing a core fucose and two galactosyl residues, Gal<sub>2</sub>GlcNAc<sub>2</sub>Man<sub>3</sub>GlcNAc<sub>2</sub>F.

The term “Man5” when used herein refers to the oligosaccharide structure shown



The term “GFI 5.0” when used herein refers to glycoengineered *Pichia pastoris* strains that produce glycoproteins having predominantly Gal<sub>2</sub>GlcNAc<sub>2</sub>Man<sub>3</sub>GlcNAc<sub>2</sub> N-glycans.

The term “GFI 6.0” when used herein refers to glycoengineered *Pichia pastoris* strains that produce glycoproteins having predominantly NANA<sub>2</sub>Gal<sub>2</sub>GlcNAc<sub>2</sub>Man<sub>3</sub>GlcNAc<sub>2</sub> N-glycans.

The term “GS5.0”, when used herein refers to the N-glycosylation structure Gal<sub>2</sub>GlcNAc<sub>2</sub>Man<sub>3</sub>GlcNAc<sub>2</sub>.

The term “GS5.5”, when used herein refers to the N-glycosylation structure NANAGal<sub>2</sub>GlcNAc<sub>2</sub>Man<sub>3</sub>GlcNAc<sub>2</sub>, which when produced in *Pichia pastoris* strains to which  $\alpha$  2,6 sialyl transferase has been glycoengineered result in  $\alpha$ 2,6-linked sialic acid and which when produced in *Pichia pastoris*

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strains to which  $\alpha$  2,3 sialyl transferase has been glycoengineered result in  $\alpha$  2,3-linked sialic acid.

The term “GS6.0”, when used herein refers to the N-glycosylation structure NANA<sub>2</sub>Gal<sub>2</sub>GlcNAc<sub>2</sub>Man<sub>3</sub>GlcNAc<sub>2</sub>, which when produced in *Pichia pastoris* strains to which  $\alpha$  2,6 sialyl transferase has been glycoengineered result in  $\alpha$  2,6-linked sialic acid and which when produced in *Pichia pastoris* strains to which  $\alpha$  2,3 sialyl transferase has been glycoengineered result in  $\alpha$  2,3-linked sialic acid.

The term “wild type” or “wt” when used herein in connection to a *Pichia pastoris* strain refers to a native *Pichia pastoris* strain that has not been subjected to genetic modification to control glycosylation.

The term “antibody”, when used herein refers to an immunoglobulin molecule capable of binding to a specific antigen through at least one antigen recognition site located in the variable region of the immunoglobulin molecule. As used herein, the term encompasses not only intact polyclonal or monoclonal antibodies, consisting of four polypeptide chains, i.e. two identical pairs of polypeptide chains, each pair having one “light” chain (LC) (about 25 kDa) and one “heavy” chain (HC) (about 50-70 kDa), but also fragments thereof, such as Fab, Fab', F(ab')<sub>2</sub>, Fv, single chain (ScFv), mutants thereof, fusion proteins comprising an antibody portion, and any other modified configuration of an immunoglobulin molecule that comprises an antigen recognition site and at least the portion of the C<sub>H</sub>2 domain of the heavy chain immunoglobulin constant region which comprises an N-linked glycosylation site of the C<sub>H</sub>2 domain, or a variant thereof. As used herein the term includes an antibody of any class, such as IgG (for example, IgG1, IgG2, IgG3 or IgG4), IgM, IgA, IgD and IgE, respectively.

The term “consensus sequence of C<sub>H</sub>2” when used herein refers to the amino acid sequence of the C<sub>H</sub>2 domain of the heavy chain constant region containing an N-linked glycosylation site which was derived from the most common amino acid sequences found in C<sub>H</sub>2 domains from a variety of antibodies.

The term “Fc region” is used to define a C-terminal region of an immunoglobulin heavy chain. The “Fc region” may be a native sequence Fc region or a variant Fc region. Although the boundaries of the Fc region of an immunoglobulin heavy chain might vary, the human IgG heavy chain Fc region is usually defined to stretch from an amino acid residue at position Cys226, or from Pro230, to the carboxyl-terminus thereof. The Fc region of an immunoglobulin comprises two constant domains, CH2 and CH3, and can optionally comprise a hinge region. In one embodiment, the Fc region comprises the amino acid sequence of SEQ ID NO:18. In one embodiment, the Fc region comprises the amino acid sequence of SEQ ID NO:19. In another embodiment, the Fc region comprises the amino acid sequence of SEQ ID NO:18, with the addition of a lysine (K) residue at the 5' end. The Fc region contains a single N-linked glycosylation site in the CH2 domain that corresponds to the Asn297 site of a full-length heavy chain of an antibody.

The term “Fc-containing polypeptide” refers to a polypeptide, such as an antibody or immunoadhesin, which comprises an Fc region. This two encompasses polypeptides comprising or consisting of (or consisting essentially of) an Fc region. Polypeptides comprising an Fc region can be generated by papain digestion of antibodies or by recombinant DNA technology.

The term “parent antibody”, “parent immunoglobulin” or “parent Fc-containing polypeptide” when used herein refers to an antibody or Fc-containing polypeptide which lacks the Fc region mutations disclosed herein. A parent Fc-containing

polypeptide may comprise a native sequence Fc region or an Fc region with pre-existing amino acid sequence modifications. A native sequence Fc region comprises an amino acid sequence identical to the amino acid sequence of an Fc region found in nature. Native sequence Fc regions include the native sequence human IgG1Fc region, the native sequence human IgG2 Fc region, the native sequence human IgG3 Fc region and the native sequence human IgG4 Fc region as well as naturally occurring variants thereof. When used as a comparator, a parent antibody or a parent Fc-containing polypeptide can be expressed in any cell. In one embodiment, the parent antibody or a parent Fc-containing polypeptide is expressed in the same cell as the Fc-containing polypeptide of the invention.

As used herein, the term “immunoadhesin” designates antibody-like molecules which combine the “binding domain” of a heterologous “adhesin” protein (e.g. a receptor, ligand or enzyme) with an immunoglobulin constant domain. Structurally, the immunoadhesins comprise a fusion of the adhesin amino acid sequence with the desired binding specificity which is other than the antigen recognition and binding site (antigen combining site) of an antibody (i.e. is “heterologous”) and an immunoglobulin constant domain sequence. The term “ligand binding domain” as used herein refers to any native cell-surface receptor or any region or derivative thereof retaining at least a qualitative ligand binding ability of a corresponding native receptor. In a specific embodiment, the receptor is from a cell-surface polypeptide having an extracellular domain that is homologous to a member of the immunoglobulin supergene family. Other receptors, which are not members of the immunoglobulin supergene family but are nonetheless specifically covered by this definition, are receptors for cytokines, and in particular receptors with tyrosine kinase activity (receptor tyrosine kinases), members of the hematopoietin and nerve growth factor which predispose the mammal to the disorder in question. In one embodiment, the disorder is cancer. Methods of making immunoadhesins are well known in the art. See, e.g., WO00/42072.

The term “Her2” or “Her2 antibody” when used herein refers to an antibody having an amino acid sequence similar to that for a commercially available Her2 antibody produced in mammalian cells, i.e. CHO cells, known as trastuzumab.

The term “*Pichia* Her2 antibody” or “*Pichia* anti-Her2 antibody” when used herein refers to an antibody having an amino acid sequence similar to that for a commercially available Her2 antibody (trastuzumab) produced in glycoengineered *Pichia pastoris*.

The term “Fc mutein antibody” when used herein refers to an antibody comprising one of the single Fc muteins or the double Fc mutein described herein.

The term “Fc mutein” when used herein refers to an Fc-containing polypeptide in which one or more point mutations have been made to the Fc region.

The term “Fc mutation” when used herein refers to a mutation made to the Fc region of an Fc-containing polypeptide. Examples of such a mutation include the F243A or V264A mutations described herein.

The term “single Fc mutein” when used herein refers to an Fc-containing polypeptide incorporating a mutation at position 243 or 264 of the Fc region. The term “F243A” refers to a mutation from F (wild-type) to A at position 243 of the Fc region of an Fc-containing polypeptide. The term “V264A” refers to a mutation from V (wild-type) to A at position 264 of the Fc region of an Fc-containing polypeptide. The position 243 and 264 represent the amino acid positions in the CH2 domain of the Fc region of an Fc-containing polypeptide.

The term “double Fc mutein” when used herein refers to an Fc-containing polypeptide comprising mutations at positions 243 and 264 of the Fc region. The term “F243A/V264A” refers to a double Fc mutein comprising the two specified mutations.

Throughout the present specification and claims, the numbering of the residues in an immunoglobulin heavy chain or an Fc-containing polypeptide is that of the EU index as in Kabat et al., Sequences of Proteins of Immunological Interest, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, Md. (1991), expressly incorporated herein by reference. The “EU index as in Kabat” refers to the residue numbering of the human IgG1 EU antibody.

The term “effector function” as used herein refers to a biochemical event that results from the interaction of an antibody Fc region with an Fc receptor or ligand. Exemplary “effector functions” include C1q binding; complement dependent cytotoxicity; Fc receptor binding; antibody-dependent cell-mediated cytotoxicity (ADCC); phagocytosis; down regulation of cell surface receptors (e.g. B cell receptor; BCR), etc. Such effector functions can be assessed using various assays known in the art.

The term “glycoengineered *Pichia pastoris*” when used herein refers to a strain of *Pichia pastoris* that has been genetically altered to express human-like N-glycans. For example, the GFI 5.0, GFI 5.5 and GFI 6.0 strains described above.

The terms “N-glycan”, “glycoprotein” and “glycoform” when used herein refer to an N-linked oligosaccharide, e.g., one that is attached by an asparagine-N-acetylglucosamine linkage to an asparagine residue of a polypeptide. Predominant sugars found on glycoproteins are glucose, galactose, mannose, fucose, N-acetylgalactosamine (GalNAc), N-acetylglucosamine (GlcNAc) and sialic acid (SA, including NANA, NGNA and derivatives and analogs thereof; including acetylated NANA or acetylated NGNA). In glycoengineered *Pichia pastoris*, sialic acid is exclusively N-acetyl-neuraminic acid (NANA) (Hamilton et al., *Science* 313 (5792): 1441-1443 (2006)). N-glycans have a common pentasaccharide core of Man<sub>3</sub>GlcNAc<sub>2</sub>, wherein “Man” refers to mannose, “Glc” refers to glucose, “NAc” refers to N-acetyl, and GlcNAc refers to N-acetylglucosamine. N-glycans differ with respect to the number of branches (antennae) comprising peripheral sugars (e.g., GlcNAc, galactose, fucose and sialic acid) that are added to the Man<sub>3</sub>GlcNAc<sub>2</sub> (“Man<sub>3</sub>”) core structure which is also referred to as the “trimannose core”, the “pentasaccharide core” or the “paucimannose core”. N-glycans are classified according to their branched constituents (e.g., high mannose, complex or hybrid).

As used herein, the term “sialic acid” or “SA” refers to any member of the sialic acid family, including without limitation: N-acetylneuraminic acid (Neu5Ac or NANA), N-glycolylneuraminic acid (NGNA) and any analog or derivative thereof (including those arising from acetylation at any position on the sialic acid molecule). Sialic acid is a generic name for a group of about 30 naturally occurring acidic carbohydrates that are essential components of a large number of glycoconjugates. Schauer, *Biochem. Society Transactions*, 11, 270-271 (1983). Sialic acids are usually the terminal residue of the oligosaccharides. N-acetylneuraminic acid (NANA) is the most common sialic acid form and N-glycolylneuraminic acid (NGNA) is the second most common form. Schauer, *Glycobiology*, 1, 449-452 (1991). NGNA is widespread throughout the animal kingdom and, according to species and tissue, often constitutes a significant proportion of the glycoconjugate-bound sialic acid. Certain species such

as chicken and man are exceptional, since they lack NGNA in normal tissues. Corfield, et al., *Cell Biology Monographs*, 10, 5-50 (1982). In human serum samples, the percentage of sialic acid in the form of NGNA is reported to be 0.01% of the total sialic acid. Schauer, "Sialic Acids as Antigenic Determinants of Complex Carbohydrates", found in *The Molecular Immunology of Complex Carbohydrates*, (Plenum Press, New York, 1988).

The term "human-like N-glycan", as used herein, refers to the N-linked oligosaccharides which closely resemble the oligosaccharides produced by non-engineered, wild-type human cells. For example, wild-type *Pichia pastoris* and other lower eukaryotic cells typically produce hypermannosylated proteins at N-glycosylation sites. The host cells described herein produce glycoproteins (for example, antibodies) comprising human-like N-glycans that are not hypermannosylated. In some embodiments, the host cells of the present invention are capable of producing human-like N-glycans with hybrid and/or complex N-glycans. The specific type of "human-like" glycans present on a specific glycoprotein produced from a host cell of the invention will depend upon the specific glycoengineering steps that are performed in the host cell.

The term "high mannose" type N-glycan when used herein refers to an N-glycan having five or more mannose residues.

The term "complex" type N-glycan when used herein refers to an N-glycan having at least one GlcNAc attached to the 1,3 mannose arm and at least one GlcNAc attached to the 1,6 mannose arm of a "trimannose" core. Complex N-glycans may also have galactose ("Gal") or N-acetylgalactosamine ("GalNAc") residues that are optionally modified with sialic acid or derivatives (e.g., "NANA" or "NeuAc", where "Neu" refers to neuraminic acid and "Ac" refers to acetyl). Complex N-glycans may also have intrachain substitutions comprising "bisecting" GlcNAc and core fucose ("Fuc"). As an example, when a N-glycan comprises a bisecting GlcNAc on the trimannose core, the structure can be represented as  $\text{Man}_3\text{GlcNAc}_2(\text{GlcNAc})$  or  $\text{Man}_3\text{GlcNAc}_3$ . When an N-glycan comprises a core fucose attached to the trimannose core, the structure may be represented as  $\text{Man}_3\text{GlcNAc}_2(\text{Fuc})$ . Complex N-glycans may also have multiple antennae on the "trimannose core," often referred to as "multiple antennary glycans."

The term "hybrid" N-glycan when used herein refers to an N-glycan having at least one GlcNAc on the terminal of the 1,3 mannose arm of the trimannose core and zero or more than one mannose on the 1,6 mannose arm of the trimannose core.

When referring to "mole percent" of a glycan present in a preparation of a glycoprotein, the term means the molar percent of a particular glycan present in the pool of N-linked oligosaccharides released when the protein preparation is treated with PNGase and then quantified by a method that is not affected by glycoform composition, (for instance, labeling a PNGase released glycan pool with a fluorescent tag such as 2-aminobenzamide and then separating by high performance liquid chromatography or capillary electrophoresis and then quantifying glycans by fluorescence intensity). For example, 50 mole percent  $\text{NANA}_2\text{Gal}_2\text{GlcNAc}_2\text{Man}_3\text{GlcNAc}_2$  means that 50 percent of the released glycans are  $\text{NANA}_2\text{Gal}_2\text{GlcNAc}_2\text{Man}_3\text{GlcNAc}_2$  and the remaining 50 percent are comprised of other N-linked oligosaccharides.

The term "anti-inflammatory antibody" as used herein, refers to an antibody intended to be used to treat inflammation. The anti-inflammatory properties of an Fc-containing polypeptide can be measured using any method known in the art. In one embodiment, the anti-inflammatory properties of

an Fc-containing polypeptide are measured using an animal model, such as the models described in Kaneko et al., *Science* 313:670-673 (2006), Anthony et al., *Science* 320:373-376 (2008), and Examples 20-21 herein. In another embodiment, the anti-inflammatory properties of an Fc-containing polypeptide are measured by determining the level of a biomarker related to inflammation (including without limitation: CRP, pro-inflammatory cytokines such as tumor necrosis factors (TNF-alpha), interferon-gamma, interleukin 6 (IL-6, IL-8, IL-10, chemokines, the coagulation marker D-dimer, sCD14, intestinal fatty acid binding peptide (IFABP), and hyaluronic acid. In one embodiment, the anti-inflammatory properties of an Fc-containing polypeptide is measured by determining the level of C-reactive protein (CRP) using a method known in the art. A decrease in the level of C-reactive protein indicates that the Fc-containing polypeptide has anti-inflammatory properties.

"Conservatively modified variants" or "conservative substitution" refers to substitutions of amino acids in a protein with other amino acids having similar characteristics (e.g. charge, side-chain size, hydrophobicity/hydrophilicity, backbone conformation and rigidity, etc.), such that the changes can frequently be made without altering the biological activity of the protein. Those of skill in this art recognize that, in general, single amino acid substitutions in non-essential regions of a polypeptide do not substantially alter biological activity (see, e.g., Watson et al. (1987) *Molecular Biology of the Gene*, The Benjamin/Cummings Pub. Co., p. 224 (4th Ed.)). In addition, substitutions of structurally or functionally similar amino acids are less likely to disrupt biological activity. Exemplary conservative substitutions are listed below:

Original residue	Conservative substitution
Ala (A)	Gly; Ser
Arg (R)	Lys; His
Asn (N)	Gln; His
Asp (D)	Glu; Asn
Cys (C)	Ser; Ala
Gln (Q)	Asn
Glu (E)	Asp; Gln
Gly (G)	Ala
His (H)	Asn; Gln
Ile (I)	Leu; Val
Leu (L)	Ile; Val
Lys (K)	Arg; His
Met (M)	Leu; Ile; Tyr
Phe (F)	Tyr; Met; Leu
Pro (P)	Ala
Ser (S)	Thr
Thr (T)	Ser
Trp (W)	Tyr; Phe
Tyr (Y)	Trp; Phe
Val (V)	Ile; Leu

Glycosylation of immunoglobulin G (IgG) in the Fc region, Asn297 (according to the EU numbering system), has been shown to be a requirement for optimal recognition and activation of effector pathways including antibody dependent cellular cytotoxicity (ADCC) and complement dependent cytotoxicity (CDC), Wright & Morrison, *Trends in Biotechnology*, 15: 26-31 (1997), Tao & Morrison, *J. Immunol.*, 143 (8):2595-2601 (1989). As such, glycosylation engineering in the constant region of IgG has become an area of active research for the development of therapeutic monoclonal antibodies (mAbs). It has been established that the presence of N-linked glycosylation at Asn297 is critical for mAb activity in immune effector function assays including ADCC, Rothman (1989), Lifely et al., *Glycobiology*, 5:813-822 (1995), Umana (1999), Shields (2002), and Shinkawa (2003), and

complement dependent cytotoxicity (CDC), Hodoniczky et al., *Biotechnol. Prog.*, 21(6): 1644-1652 (2005), and Jefferis et al., *Chem. Immunol.*, 65: 111-128 (1997). This effect on function has been attributed to the specific conformation adopted by the glycosylated Fc domain, which appears to be lacking when glycosylation is absent. More specifically, IgG which lacks glycosylation in the Fc C<sub>H</sub>2 domain does not bind to FcγR, including FcγRI, FcγRII, and FcγRIII, Rothman (1989).

Not only does the presence of glycosylation appear to play a role in the effector function of an antibody, the particular composition of the N-linked oligosaccharide is also important. For example, the presence of fucose shows a marked effect on in vitro FcγRIIIa binding and in vitro ADCC, Rothman (1989), and Li et al., *Nat. Biotechnol.* 24(2): 2100-2115 (2006). Recombinant antibodies produced by mammalian cell culture, such as CHO or NS0, contain N-linked oligosaccharides that are predominately fucosylated, Hossler et al., *Biotechnology and Bioengineering*, 95(5):946-960 (2006), Umana (1999), and Jefferis et al., *Biotechnol. Prog.* 21:11-16 (2005). Additionally, there is evidence that sialylation in the Fc region may impart anti-inflammatory properties to antibodies. Intravenous immunoglobulin (IVIg) purified over a lectin column to enrich for the sialylated form showed a distinct anti-inflammatory effect limited to the sialylated Fc fragment and was linked to an increase in expression of the inhibitory receptor FcγRIIb, Nimmerjahn and Ravetch., *J. Exp. Med.* 204:11-15 (2007).

Glycosylation in the Fc region of an antibody derived from mammalian cell lines typically consists of a heterogeneous mix of glycoforms, with the predominant forms typically being comprised of the complex fucosylated glycoforms: G0F, G1F, and, to a lesser extent, G2F. Possible conditions resulting in incomplete galactose transfer to the G0F structure include, but are not limited to, non-optimized galactose transfer machinery, such as β-1,4 galactosyl transferase, and poor UDP-galactose transport into the Golgi apparatus, suboptimal cell culture and protein expression conditions, and steric hindrance by amino acid residues neighboring the oligosaccharide. While each of these conditions may modulate the ultimate degree of terminal galactose, it is thought that subsequent sialic acid transfer to the Fc oligosaccharide is inhibited by the closed pocket configuration of the C<sub>H</sub>2 domain. See, for example, FIG. 1, Jefferis, R., *Nature Biotech.*, 24 (10): 1230-1231, 2006. Without the correct terminal monosaccharide, specifically galactose, or with insufficient terminal galactosylated forms, there is little possibility of producing a sialylated form, capable of acting as a therapeutic protein, even when produced in the presence of sialyl transferase. Protein engineering and structural analysis of human IgG-Fc glycoforms has shown that glycosylation profiles are affected by Fc conformation, such as the finding that increased levels of galactose and sialic acid on oligosaccharides derived from CHO-produced IgG3 could be achieved when specific amino acids from the Fc pocket were mutated, to an alanine including F241, F243, V264, D265 and R301. Lund et al., *J. Immunol.* 157(11); 4963-4969 (1996). It was further shown that certain mutations had some effect on cell mediated superoxide generation and complement mediated red cell lysis, which are used as surrogate markers for FcγRI and C1q binding, respectively.

It has been reported that yeast have been genetically engineered to produce host strains capable of secreting glycoproteins with highly uniform glycosylation. Choi et al., *PNAS, USA* 100(9): 5022-5027 (2003) describes the use of libraries of α 1,2 mannosidase catalytic domains and N-acetylglucosaminyltransferase I catalytic domains in combination with

a library of fungal type II membrane protein leader sequences to localize the catalytic domains to the secretory pathway. In this way, strains were isolated that produced in vivo glycoproteins with uniform Man<sub>5</sub>GlcNAc<sub>2</sub> or GlcNAcMan<sub>5</sub>GlcNAc<sub>2</sub> N-glycan structures. Hamilton et al., *Science* 313 (5792): 1441-1443 (2006) described the production of a glycoprotein, erythropoietin, produced in *Pichia pastoris*, as having a glycan composition that consisted predominantly of a bisialylated glycan structure, GS6.0, NANA<sub>2</sub>Gal<sub>2</sub>GlcNAc<sub>2</sub>Man<sub>3</sub>GlcNAc<sub>2</sub> (90.5%) and monosialylated, GS5.5, NANAGal<sub>2</sub>GlcNAc<sub>2</sub>Man<sub>3</sub>GlcNAc<sub>2</sub> (7.9%). However, an antibody produced in a similar strain will have a markedly lower content of sialylated N-glycan due to the relatively low level of terminal galactose substrate in the antibody as seen in FIG. 4. It has also recently been shown that sialylation of a Fc oligosaccharide imparts anti-inflammatory properties on therapeutic intravenous gamma globulin and its Fc fragments, Kaneko et al., *Science* 313(5787): 670-673 (2006), and that the anti-inflammatory activity is dependent on the α 2,6-linked form, but not the α 2,3 form, of sialic acid, Anthony et al., *Science*, 320: 373-376 (2008).

#### Host Organisms and Cell Lines

The Fc-containing polypeptides of this invention can be made in any host organism or cell line. In one embodiment, an Fc-containing polypeptide of the invention is made in a host cell which is capable of producing sialylated N-glycans.

In one embodiment, an Fc-containing polypeptide of the invention is made in a mammalian cell where the cell either endogenously or through genetic or process manipulation produces glycoproteins containing either a mixture of terminal α2-6 and α2-3 sialic acid, or only terminal α2-6 sialic acid. The propagation of mammalian cells in culture (tissue culture) has become a routine procedure. Examples of useful mammalian host cell lines are monkey kidney CV1 line transformed by SV40 (COS-7, ATCC CRL 1651); human embryonic kidney line (293 or 293 cells subcloned for growth in suspension culture); baby hamster kidney cells (BHK, ATCC CCL 10); Chinese hamster ovary cells/-DHFR (CHO); mouse sertoli cells (TM4.); monkey kidney cells (CV1 ATCC CCL 70); African green monkey kidney cells (VERO-76, ATCC CRL-1587); human cervical carcinoma cells (HFLA, ATCC CCL 2); canine kidney cells (MDCK, ATCC CCL 34); buffalo rat liver cells (BRL 3A, ATCC CRL 1442); human lung cells (W138, ATCC CCL 75); human liver cells (Hep G2, FIB 8065); mouse mammary tumor (MMT 060562, ATCC CCL51); TRI cells; MRC 5 cells; FS4 cells; hybridoma cell lines; NS0; SP2/0; and a human hepatoma line (Hep G2).

In one embodiment, an Fc-containing polypeptide of the invention can be made in a plant cell which is engineered to produce sialylated N-glycans. See, e.g., Cox et al., *Nature Biotechnology* (2006) 24, 1591-1597 (2006) and Castilho et al., *J. Biol. Chem.* 285(21): 15923-15930 (2010).

In one embodiment, an Fc-containing polypeptide of the invention can be made in an insect cell which is engineered to produce sialylated N-glycans. See, e.g., Harrison and Jarvis, *Adv. Virus Res.* 68:159-91 (2006).

In one embodiment, an Fc-containing polypeptide of the invention can be made in a bacterial cell which is engineered to produce sialylated N-glycans. See, e.g., Lizak et al., *Bioconjugate Chem.* 22:488-496 (2011).

In one embodiment, an Fc-containing polypeptide of the invention can be made in a lower eukaryotic host cell or organism. Recent developments allow the production of fully humanized therapeutics in lower eukaryotic host organisms, yeast and filamentous fungi, such as *Pichia pastoris*, Gerngross et al., U.S. Pat. Nos. 7,029,872 and 7,449,308, the

disclosures of which are hereby incorporated by reference. See also Jacobs et al., *Nature Protocols* 4(1):58-70 (2009). Applicants herein have further developed modified *Pichia pastoris* host organisms and cell lines capable of expressing antibodies comprising two mutations to the amino acids at positions 243 and 264 in the Fc region of the heavy chain. The antibodies having these mutations had increased levels and a more homogeneous composition of the  $\alpha$  2,6-linked sialylated N-glycans when compared to a parent antibody. Applicants have also surprisingly found that mutations at amino acids at positions 243 and 264 in the Fc region of the heavy chain resulted in an antibody which had decreased binding to all Fc $\gamma$  receptors and decreased C1q binding, the former of which is a surrogate for ADCC, which was independent of the increased levels of the  $\alpha$  2,6-linked sialic acid. Thus, based on the increased level and more homogeneity of the terminal  $\alpha$  2,6-linked sialic acid N-glycan, those of ordinary skill in the art would recognize and appreciate that the materials and methods described herein can be used to produce recombinant glycosylated antibodies in lower eukaryotic cells, such as yeast and filamentous fungi, and, in particular, *Pichia pastoris*, that have enhanced anti-inflammatory properties when compared to a parent antibody.

Due to the decreased Fc $\gamma$ R and C1q binding, the materials and methods described herein can be used to produce recombinant glycosylated antibodies with decreased effector function when compared to a parent antibody. Antibodies so produced in *Pichia pastoris* by the methods of the invention were produced at high yield, with decreased effector function, and had a predominant species of glycoprotein having a terminal  $\alpha$  2,6-linked sialic acid residue as compared to antibodies produced in glycoengineered *Pichia pastoris* cells lacking the specific Fc mutations or in *Pichia pastoris* host cells retaining their endogenous glycosylation machinery.

In one embodiment, an Fc-containing polypeptide of the invention is made in a host cell, more preferably a yeast or filamentous fungal host cell, that has been engineered to produce glycoproteins having a predominant N-glycan comprising a terminal sialic acid. In one embodiment of the invention, the predominant N-glycan is the  $\alpha$  2,6 linked form of SA<sub>2</sub>Gal<sub>2</sub>GlcNAc<sub>2</sub>Man<sub>3</sub>GlcNAc<sub>2</sub>, produced in strains glycoengineered with  $\alpha$  2,6 sialyl transferase which do not produce any  $\alpha$  2,3 linked sialic acid. In other embodiments, the strain will be engineered to express an  $\alpha$  2,3 sialyl transferase alone or in combination with an  $\alpha$  2,6, sialyl transferase, resulting in  $\alpha$  2,3 linked or a combination of  $\alpha$  2,6 and  $\alpha$  2,3 linked sialic acid as the predominant N-glycans.

The cell lines to be used to make the Fc-containing polypeptides of the invention can be any cell line, in particular cell lines with the capability of producing one or more sialylated glycoproteins. Those of ordinary skill in the art would recognize and appreciate that the materials and methods described herein are not limited to the specific strain of *Pichia pastoris* provided as an example herein, but could include any *Pichia pastoris* strain or other yeast or filamentous fungal strains in which N-glycans with one or more terminal galactose, such as Gal<sub>2</sub>GlcNAc<sub>2</sub>Man<sub>3</sub>, are produced. The terminal galactose acts as a substrate for the production of  $\alpha$  2,6-linked sialic acid, resulting in the N-glycan structure NANA<sub>2</sub>Gal<sub>2</sub>GlcNAc<sub>2</sub>Man<sub>3</sub>GlcNAc<sub>2</sub>. Examples of suitable strains are described in U.S. Pat. No. 7,029,872, US 2006-0286637 and Hamilton et al., *Science* 313 (5792): 1441-1443 (2006), the descriptions of which are incorporated herein as if set forth at length.

In general, lower eukaryotes such as yeast are used for expression of the proteins, particularly glycoproteins because they can be economically cultured, give high yields, and

when appropriately modified are capable of suitable glycosylation. Yeast particularly offers established genetics allowing for rapid transformations, tested protein localization strategies and facile gene knock-out techniques. Suitable vectors have expression control sequences, such as promoters, including 3-phosphoglycerate kinase or other glycolytic enzymes, and an origin of replication, termination sequences and the like as desired.

While the invention has been demonstrated herein using the methylotrophic yeast *Pichia pastoris*, other useful lower eukaryote host cells include *Pichia pastoris*, *Pichia finlandica*, *Pichia trehalophila*, *Pichia koclamae*, *Pichia membranaefaciens*, *Pichia minuta* (*Ogataea minuta*, *Pichia lindneri*), *Pichia opuntiae*, *Pichia thermotolerans*, *Pichia salictaria*, *Pichia guercuum*, *Pichia pijperi*, *Pichia stiptis*, *Pichia methanolica*, *Pichia* sp., *Saccharomyces cerevisiae*, *Saccharomyces* sp., *Hansenula polymorphs*, *Kluyveromyces* sp., *Kluyveromyces lactis*, *Candida albicans*, *Aspergillus nidulans*, *Aspergillus niger*, *Aspergillus oryzae*, *Trichoderma reesei*, *Chrysosporium lucknowense*, *Fusarium* sp., *Fusarium gramineum*, *Fusarium venenatum* and *Neurospora crassa*. Various yeasts, such as *K. lactis*, *Pichia pastoris*, *Pichia methanolica*, and *Hansenula polymorpha* are particularly suitable for cell culture because they are able to grow to high cell densities and secrete large quantities of recombinant protein. Likewise, filamentous fungi, such as *Aspergillus niger*, *Fusarium* sp, *Neurospora crassa* and others can be used to produce glycoproteins of the invention at an industrial scale.

Lower eukaryotes, particularly yeast and filamentous fungi, can be genetically modified so that they express glycoproteins in which the glycosylation pattern is human-like or humanized. As indicated above, the term "human-like N-glycan", as used herein refers, to the N-linked oligosaccharides which closely resemble the oligosaccharides produced by non-engineered, wild-type human cells. In preferred embodiments of the present invention, the host cells of the present invention are capable of producing human-like glycoproteins with hybrid and/or complex N-glycans; i.e., "human-like N-glycosylation." The specific "human-like" glycans predominantly present on glycoproteins produced from the host cells of the invention will depend upon the specific engineering steps that are performed. In this manner, glycoprotein compositions can be produced in which a specific desired glycoform is predominant in the composition. Such can be achieved by eliminating selected endogenous glycosylation enzymes and/or genetically engineering the host cells and/or supplying exogenous enzymes to mimic all or part of the mammalian glycosylation pathway as described in U.S. Pat. No. 7,449,308. If desired, additional genetic engineering of the glycosylation can be performed, such that the glycoprotein can be produced with or without core fucosylation. Use of lower eukaryotic host cells is further advantageous in that these cells are able to produce highly homogeneous compositions of glycoprotein, such that the predominant glycoform of the glycoprotein may be present as greater than thirty mole percent of the glycoprotein in the composition. In particular aspects, the predominant glycoform may be present in greater than forty mole percent, fifty mole percent, sixty mole percent, seventy mole percent and, most preferably, greater than eighty mole percent of the glycoprotein present in the composition.

Lower eukaryotes, particularly yeast, can be genetically modified so that they express glycoproteins in which the glycosylation pattern is human-like or humanized. Such can be achieved by eliminating selected endogenous glycosylation enzymes and/or supplying exogenous enzymes as

described by Gerngross et al., U.S. Pat. No. 7,449,308. For example, a host cell can be selected or engineered to be depleted in  $\alpha$ 1,6-mannosyl transferase activities, which would otherwise add mannose residues onto the N-glycan on a glycoprotein.

In one embodiment, the host cell further includes an  $\alpha$ 1,2-mannosidase catalytic domain fused to a cellular targeting signal peptide not normally associated with the catalytic domain and selected to target the  $\alpha$ 1,2-mannosidase activity to the ER or Golgi apparatus of the host cell. Passage of a recombinant glycoprotein through the ER or Golgi apparatus of the host cell produces a recombinant glycoprotein comprising a  $\text{Man}_5\text{GlcNAc}_2$  glycoform, for example, a recombinant glycoprotein composition comprising predominantly a  $\text{Man}_5\text{GlcNAc}_2$  glycoform. For example, U.S. Pat. Nos. 7,029,872 and 7,449,308 and U.S. Published Patent Application No. 2005/0170452 disclose lower eukaryote host cells capable of producing a glycoprotein comprising a  $\text{Man}_5\text{GlcNAc}_2$  glycoform.

In a further embodiment, the immediately preceding host cell further includes a GlcNAc transferase I (GnT I) catalytic domain fused to a cellular targeting signal peptide not normally associated with the catalytic domain and selected to target GlcNAc transferase I activity to the ER or Golgi apparatus of the host cell. Passage of the recombinant glycoprotein through the ER or Golgi apparatus of the host cell produces a recombinant glycoprotein comprising a  $\text{GlcNAcMan}_5\text{GlcNAc}_2$  glycoform, for example a recombinant glycoprotein composition comprising predominantly a  $\text{GlcNAcMan}_5\text{GlcNAc}_2$  glycoform. U.S. Pat. Nos. 7,029,872 and 7,449,308 and U.S. Published Patent Application No. 2005/0170452 disclose lower eukaryote, host cells capable of producing a glycoprotein comprising a  $\text{GlcNAcMan}_5\text{GlcNAc}_2$  glycoform. The glycoprotein produced in the above cells can be treated in vitro with a hexosaminidase to produce a recombinant glycoprotein comprising a  $\text{Man}_5\text{GlcNAc}_2$  glycoform.

In a further embodiment, the immediately preceding host cell further includes a mannosidase II catalytic domain fused to a cellular targeting signal peptide not normally associated with the catalytic domain and selected to target mannosidase II activity to the ER or Golgi apparatus of the host cell. Passage of the recombinant glycoprotein through the ER or Golgi apparatus of the host cell produces a recombinant glycoprotein comprising a  $\text{GlcNAcMan}_3\text{GlcNAc}_2$  glycoform, for example a recombinant glycoprotein composition comprising predominantly a  $\text{GlcNAcMan}_3\text{GlcNAc}_2$  glycoform. U.S. Pat. No. 7,029,872 and U.S. Published Patent Application No. 2004/0230042 disclose lower eukaryote host cells that express mannosidase II enzymes and are capable of producing glycoproteins having predominantly a  $\text{GlcNAcMan}_3\text{GlcNAc}_2$  glycoform. The glycoprotein produced in the above cells can be treated in vitro with a hexosaminidase to produce a recombinant glycoprotein comprising a  $\text{Man}_3\text{GlcNAc}_2$  glycoform.

In a further embodiment, the immediately preceding host cell further includes GlcNAc transferase II (GnT II) catalytic domain fused to a cellular targeting signal peptide not normally associated with the catalytic domain and selected to target GlcNAc transferase II activity to the ER or Golgi apparatus of the host cell. Passage of the recombinant glycoprotein through the ER or Golgi apparatus of the host cell produces a recombinant glycoprotein comprising a  $\text{GlcNAc}_2\text{Man}_3\text{GlcNAc}_2$  glycoform, for example a recombinant glycoprotein composition comprising predominantly a  $\text{GlcNAc}_2\text{Man}_3\text{GlcNAc}_2$  glycoform. U.S. Pat. Nos. 7,029,872 and 7,449,308 and U.S. Published Patent Application No.

2005/0170452 disclose lower eukaryote host cells capable of producing a glycoprotein comprising a  $\text{GlcNAc}_2\text{Man}_3\text{GlcNAc}_2$  glycoform. The glycoprotein produced in the above cells can be treated in vitro with a hexosaminidase to produce a recombinant glycoprotein comprising a  $\text{Man}_3\text{GlcNAc}_2$  glycoform.

In a further embodiment, the immediately preceding host cell further includes a galactosyltransferase catalytic domain fused to a cellular targeting signal peptide not normally associated with the catalytic domain and selected to target galactosyltransferase activity to the ER or Golgi apparatus of the host cell. Passage of the recombinant glycoprotein through the ER or Golgi apparatus of the host cell produces a recombinant glycoprotein comprising a  $\text{GalGlcNAc}_2\text{Man}_3\text{GlcNAc}_2$  or  $\text{Gal}_2\text{GlcNAc}_2\text{Man}_3\text{GlcNAc}_2$  glycoform, or mixture thereof for example a recombinant glycoprotein composition comprising predominantly a  $\text{GalGlcNAc}_2\text{Man}_3\text{GlcNAc}_2$  glycoform or  $\text{Gal}_2\text{GlcNAc}_2\text{Man}_3\text{GlcNAc}_2$  glycoform or mixture thereof. U.S. Pat. No. 7,029,872 and U.S. Published Patent Application No. 2006/0040353 discloses lower eukaryote host cells capable of producing a glycoprotein comprising a  $\text{Gal}_2\text{GlcNAc}_2\text{Man}_3\text{GlcNAc}_2$  glycoform. The glycoprotein produced in the above cells can be treated in vitro with a galactosidase to produce a recombinant glycoprotein comprising a  $\text{GlcNAc}_2\text{Man}_3\text{GlcNAc}_2$  glycoform, for example a recombinant glycoprotein composition comprising predominantly a  $\text{GlcNAc}_2\text{Man}_3\text{GlcNAc}_2$  glycoform.

In a further embodiment, the immediately preceding host cell further includes a sialyltransferase catalytic domain fused to a cellular targeting signal peptide not normally associated with the catalytic domain and selected to target sialyltransferase activity to the ER or Golgi apparatus of the host cell. In a preferred embodiment, the sialyltransferase is an  $\alpha$ 2,6-sialyltransferase. Passage of the recombinant glycoprotein through the ER or Golgi apparatus of the host cell produces a recombinant glycoprotein comprising predominantly a  $\text{NANA}_2\text{Gal}_2\text{GlcNAc}_2\text{Man}_3\text{GlcNAc}_2$  glycoform or  $\text{NANAGal}_2\text{GlcNAc}_2\text{Man}_3\text{GlcNAc}_2$  glycoform or mixture thereof. For lower eukaryote host cells such as yeast and filamentous fungi, it is useful that the host cell further include a means for providing CMP-sialic acid for transfer to the N-glycan. U.S. Published Patent Application No. 2005/0260729 discloses a method for genetically engineering lower eukaryotes to have a CMP-sialic acid synthesis pathway and U.S. Published Patent Application No. 2006/0286637 discloses a method for genetically engineering lower eukaryotes to produce sialylated glycoproteins. To enhance the amount of sialylation, it can be advantageous to construct the host cell to include two or more copies of the CMP-sialic acid synthesis pathway or two or more copies of the sialyltransferase. The glycoprotein produced in the above cells can be treated in vitro with a neuraminidase to produce a recombinant glycoprotein comprising predominantly a  $\text{Gal}_2\text{GlcNAc}_2\text{Man}_3\text{GlcNAc}_2$  glycoform or  $\text{GalGlcNAc}_2\text{Man}_3\text{GlcNAc}_2$  glycoform or mixture thereof.

Any one of the preceding host cells can further include one or more GlcNAc transferase selected from the group consisting of GnT III, GnT IV, GnT V, GnT VI, and GnT IX to produce glycoproteins having bisected (GnT III) and/or multiantennary (GnT IV, V, VI, and IX) N-glycan structures such as disclosed in U.S. Published Patent Application Nos. 2005/0208617 and 2007/0037248. Further, the preceding host cells can produce recombinant glycoproteins (for example, antibodies) comprising  $\text{SA}(1-4)\text{Gal}(1-4)\text{GlcNAc}(2-4)\text{Man}_3\text{GlcNAc}_2$ , including antibodies comprising  $\text{NANA}(1-4)\text{Gal}(1-4)\text{GlcNAc}(2-4)\text{Man}_3\text{GlcNAc}_2$ ,  $\text{NGNA}(1-4)\text{Gal}$



(1-4)GlcNAc(2-4)Man<sub>3</sub>GlcNAc<sub>2</sub> or a combination of NANA (1-4)Gal(1-4)GlcNAc(2-4) Man<sub>3</sub>GlcNAc<sub>2</sub> and NGNA(1-4)Gal(1-4)GlcNAc(2-4) Man<sub>3</sub>GlcNAc<sub>2</sub>. In one embodiment, the recombinant glycoprotein will comprise N-glycans comprising a structure selected from the group consisting of SA(1-4)Gal(1-4)GlcNAc(2-4) Man<sub>3</sub>GlcNAc<sub>2</sub> and devoid of any  $\alpha$ 2-3 linked SA.

In further embodiments, the host cell that produces glycoproteins that have predominantly GlcNAcMan<sub>5</sub>GlcNAc<sub>2</sub> N-glycans further includes a galactosyltransferase catalytic domain fused to a cellular targeting signal peptide not normally associated with the catalytic domain and selected to target the galactosyltransferase activity to the ER or Golgi apparatus of the host cell. Passage of the recombinant glycoprotein through the ER or Golgi apparatus of the host cell produces a recombinant glycoprotein comprising predominantly the GalGlcNAcMan<sub>5</sub>GlcNAc<sub>2</sub> glycoform.

In a further embodiment, the immediately preceding host cell that produced glycoproteins that have predominantly the GalGlcNAcMan<sub>5</sub>GlcNAc<sub>2</sub> N-glycans further includes a sialyltransferase catalytic domain fused to a cellular targeting signal peptide not normally associated with the catalytic domain and selected to target sialyltransferase activity to the ER or Golgi apparatus of the host cell. Passage of the recombinant glycoprotein through the ER or Golgi apparatus of the host cell produces a recombinant glycoprotein comprising a SAGalGlcNAcMan<sub>5</sub>GlcNAc<sub>2</sub> glycoform (for example NANAGalGlcNAcMan<sub>5</sub>GlcNAc<sub>2</sub> or NGNAGalGlcNAcMan<sub>5</sub>GlcNAc<sub>2</sub> or a mixture thereof).

Any of the preceding host cells can further include one or more sugar transporters such as UDP-GlcNAc transporters (for example, *Kluyveromyces lactis* and *Mus musculus* UDP-GlcNAc transporters), UDP-galactose transporters (for example, *Drosophila melanogaster* UDP-galactose transporter), and CMP-sialic acid transporter (for example, human sialic acid transporter). Because lower eukaryote host cells such as yeast and filamentous fungi lack the above transporters, it is preferable that lower eukaryote host cells such as yeast and filamentous fungi be genetically engineered to include the above transporters.

Further, any of the preceding host cells can be further manipulated to increase N-glycan occupancy. See e, g., Gaultitzek et al., *Biotechnol. Bioengin.* 103:1164-1175 (2009); Jones et al., *Biochim. Biospyhs. Acta* 1726:121-137 (2005); WO2006/107990. In one embodiment, any of the preceding host cells can be further engineered to comprise at least one nucleic acid molecule encoding a heterologous single-subunit oligosaccharyltransferase (for example, *Leishmania* sp. STT3A protein, STT3B protein, STT3C protein, STT3D protein or combinations thereof) and a nucleic acid molecule encoding the heterologous glycoprotein, and wherein the host cell expresses the endogenous host cell genes encoding the proteins comprising the endogenous OTase complex. In one embodiment, any of the preceding host cells can be further engineered to comprise at least one nucleic acid molecule encoding a *Leishmania* sp. STT3D protein and a nucleic acid molecule encoding the heterologous glycoprotein, and wherein the host cell expresses the endogenous host cell genes encoding the proteins comprising the endogenous OTase complex.

Host cells further include lower eukaryote cells (e.g., yeast such as *Pichia pastoris*) that are genetically engineered to produce glycoproteins that do not have  $\alpha$ -mannosidase-resistant N-glycans. This can be achieved by deleting or disrupting one or more of the  $\beta$ -mannosyltransferase genes (e.g., BMT1, BMT2, BMT3, and BMT4) (See, U.S. Published Patent Application No. 2006/0211085) and glycoproteins having

phosphomannose residues by deleting or disrupting one or both of the phosphomannosyl transferase genes PNO1 and MNN4B (See for example, U.S. Pat. Nos. 7,198,921 and 7,259,007), which in further aspects can also include deleting or disrupting the MNN4A gene. Disruption includes disrupting the open reading frame encoding the particular enzymes or disrupting expression of the open reading frame or abrogating translation of RNAs encoding one or more of the  $\beta$ -mannosyltransferases and/or phosphomannosyltransferases using interfering RNA, antisense RNA, or the like. Further, cells can produce glycoproteins with  $\alpha$ -mannosidase-resistant N-glycans through the addition of chemical inhibitors or through modification of the cell culture condition. These host cells can be further modified as described above to produce particular N-glycan structures.

Host cells further include lower eukaryote cells (e.g., yeast such as *Pichia pastoris*) that are genetically modified to control O-glycosylation of the glycoprotein by deleting or disrupting one or more of the protein O-mannosyltransferase (Dol-P-Man:Protein (Ser/Thr) Mannosyl Transferase genes) (PMTs) (See U.S. Pat. No. 5,714,377) or grown in the presence of Pmtp inhibitors and/or an  $\alpha$ -mannosidase as disclosed in Published International Application No. WO 2007/061631, or both. Disruption includes disrupting the open reading frame encoding the Pmtp or disrupting expression of the open reading frame or abrogating translation of RNAs encoding one or more of the Pmtps using interfering RNA, antisense RNA, or the like. The host cells can further include any one of the aforementioned host cells modified to produce particular N-glycan structures.

Pmtp inhibitors include but are not limited to a benzylidene thiazolidinediones. Examples of benzylidene thiazolidinediones that can be used are 5-[[3,4-bis(phenylmethoxy)phenyl]methylene]-4-oxo-2-thioxo-3-thiazolidineacetic Acid; 5-[[3-(1-Phenylethoxy)-4-(2-phenylethoxy)]phenyl]methylene]-4-oxo-2-thioxo-3-thiazolidineacetic Acid; and 5-[[3-(1-Phenyl-2-hydroxy)ethoxy)-4-(2-phenylethoxy)]phenyl]methylene]-4-oxo-2-thioxo-3-thiazolidineacetic acid.

In particular embodiments, the function or expression of at least one endogenous PMT gene is reduced, disrupted, or deleted. For example, in particular embodiments the function or expression of at least one endogenous PMT gene selected from the group consisting of the PMT1, PMT2, PMT3, and PMT4 genes is reduced, disrupted, or deleted; or the host cells are cultivated in the presence of one or more PMT inhibitors. In further embodiments, the host cells include one or more PMT gene deletions or disruptions and the host cells are cultivated in the presence of one or more Pmtp inhibitors. In particular aspects of these embodiments, the host cells also express a secreted  $\alpha$ -1,2-mannosidase.

PMT deletions or disruptions and/or Pmtp inhibitors control O-glycosylation by reducing O-glycosylation occupancy, that is, by reducing the total number of O-glycosylation sites on the glycoprotein that are glycosylated. The further addition of an  $\alpha$ -1,2-mannosidase that is secreted by the cell controls O-glycosylation by reducing the mannose chain length of the O-glycans that are on the glycoprotein. Thus, combining PMT deletions or disruptions and/or Pmtp inhibitors with expression of a secreted  $\alpha$ -1,2-mannosidase controls O-glycosylation by reducing occupancy and chain length. In particular circumstances, the particular combination of PMT deletions or disruptions, Pmtp inhibitors, and  $\alpha$ -1,2-mannosidase is determined empirically as particular heterologous glycoproteins (Fabs and antibodies, for example) may be expressed and transported through the Golgi apparatus with different degrees of efficiency and thus may require a particular combination of PMT deletions or disruptions, Pmtp

inhibitors, and  $\alpha$ -1,2-mannosidase. In another aspect, genes encoding one or more endogenous mannosyltransferase enzymes are deleted. This deletion(s) can be in combination with providing the secreted  $\alpha$ -1,2-mannosidase and/or PMT inhibitors or can be in lieu of providing the secreted  $\alpha$ -1,2-mannosidase and/or PMT inhibitors.

Thus, the control of O-glycosylation can be useful for producing particular glycoproteins in the host cells disclosed herein in better total yield or in yield of properly assembled glycoprotein. The reduction or elimination of O-glycosylation appears to have a beneficial effect on the assembly and transport of whole antibodies and Fab fragments as they traverse the secretory pathway and are transported to the cell surface. Thus, in cells in which O-glycosylation is controlled, the yield of properly assembled antibodies or Fab fragments is increased over the yield obtained in host cells in which O-glycosylation is not controlled.

To reduce or eliminate the likelihood of N-glycans and O-glycans with  $\beta$ -linked mannose residues, which are resistant to  $\alpha$ -mannosidases, the recombinant glycoengineered *Pichia pastoris* host cells are genetically engineered to eliminate glycoproteins having  $\alpha$ -mannosidase-resistant N-glycans by deleting or disrupting one or more of the  $\beta$ -mannosyltransferase genes (e.g., BMT1, BMT2, BMT3, and BMT4) (See, U.S. Pat. Nos. 7,465,577 and 7,713,719). The deletion or disruption of BMT2 and one or more of BMT1, BMT3, and BMT4 also reduces or eliminates detectable cross reactivity to antibodies against host cell protein.

Yield of glycoprotein can in some situations be improved by overexpressing nucleic acid molecules encoding mammalian or human chaperone proteins or replacing the genes encoding one or more endogenous chaperone proteins with nucleic acid molecules encoding one or more mammalian or human chaperone proteins. In addition, the expression of mammalian or human chaperone proteins in the host cell also appears to control O-glycosylation in the cell. Thus, further included are the host cells herein wherein the function of at least one endogenous gene encoding a chaperone protein has been reduced or eliminated, and a vector encoding at least one mammalian or human homolog of the chaperone protein is expressed in the host cell. Also included are host cells in which the endogenous host cell chaperones and the mammalian or human chaperone proteins are expressed. In further aspects, the lower eukaryotic host cell is a yeast or filamentous fungi host cell. Examples of the use of chaperones of host cells in which human chaperone proteins are introduced to improve the yield and reduce or control O-glycosylation of recombinant proteins has been disclosed in Published International Application No. WO 2009105357 and WO2010019487 (the disclosures of which are incorporated herein by reference). Like above, further included are lower eukaryotic host cells wherein, in addition to replacing the genes encoding one or more of the endogenous chaperone proteins with nucleic acid molecules encoding one or more mammalian or human chaperone proteins or overexpressing one or more mammalian or human chaperone proteins as described above, the function or expression of at least one endogenous gene encoding a protein O-mannosyltransferase (PMT) protein is reduced, disrupted, or deleted. In particular embodiments, the function of at least one endogenous PMT gene selected from the group consisting of the PMT1, PMT2, PMT3, and PMT4 genes is reduced, disrupted, or deleted.

In addition, O-glycosylation may have an effect on an antibody or Fab fragment's affinity and/or avidity for an antigen. This can be particularly significant when the ultimate host cell for production of the antibody or Fab is not the same as the host cell that was used for selecting the antibody. For

example, O-glycosylation might interfere with an antibody's or Fab fragment's affinity for an antigen, thus an antibody or Fab fragment that might otherwise have high affinity for an antigen might not be identified because O-glycosylation may interfere with the ability of the antibody or Fab fragment to bind the antigen. In other cases, an antibody or Fab fragment that has high avidity for an antigen might not be identified because O-glycosylation interferes with the antibody's or Fab fragment's avidity for the antigen. In the preceding two cases, an antibody or Fab fragment that might be particularly effective when produced in a mammalian cell line might not be identified because the host cells for identifying and selecting the antibody or Fab fragment was of another cell type, for example, a yeast or fungal cell (e.g., a *Pichia pastoris* host cell). It is well known that O-glycosylation in yeast can be significantly different from O-glycosylation in mammalian cells. This is particularly relevant when comparing wild type yeast O-glycosylation with mucin-type or dystroglycan type O-glycosylation in mammals. In particular cases, O-glycosylation might enhance the antibody or Fab fragments affinity or avidity for an antigen instead of interfere with antigen binding. This effect is undesirable when the production host cell is to be different from the host cell used to identify and select the antibody or Fab fragment (for example, identification and selection is done in yeast and the production host is a mammalian cell) because in the production host the O-glycosylation will no longer be of the type that caused the enhanced affinity or avidity for the antigen. Therefore, controlling O-glycosylation can enable use of the materials and methods herein to identify and select antibodies or Fab fragments with specificity for a particular antigen based upon affinity or avidity of the antibody or Fab fragment for the antigen without identification and selection of the antibody or Fab fragment being influenced by the O-glycosylation system of the host cell. Thus, controlling O-glycosylation further enhances the usefulness of yeast or fungal host cells to identify and select antibodies or Fab fragments that will ultimately be produced in a mammalian cell line.

Those of ordinary skill in the art would further appreciate and understand how to utilize the methods and materials described herein in combination with other *Pichia pastoris* and yeast cell lines that have been genetically engineered to produce specific N-glycans or sialylated glycoproteins, such as, but, not limited to, the host organisms and cell lines described above that have been genetically engineered to produce specific galactosylated or sialylated forms. See, for example, US 2006-0286637, Production of Sialylated N-Glycans in Lower Eukaryotes, in which the pathway for galactose uptake and utilization as a carbon source has been genetically modified, the description of which is incorporated herein as if set forth at length.

Additionally, the methods herein can be used to produce the above described recombinant Fc-containing polypeptides in other lower eukaryotic cell lines which have been engineered to produce human-like and human glycoproteins that do not have  $\alpha$  2,6 sialyltransferase activity. The methods can also be used to produce the above described recombinant Fc-containing polypeptides in eukaryotic cell lines in which production of sialylated N-glycans is an innate feature.

Levels of  $\alpha$  2,3 and  $\alpha$  2,6 linked sialic acid on the Fc-containing polypeptides can be measured using well known techniques including nuclear magnetic resonance (NMR), normal phase high performance liquid chromatography (HPLC), and high performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD).

## Biological Properties of Fc Muteins

For many Fc-containing polypeptides the lack of or significant decrease in effector function, as shown by decreased FcγR and C1q binding, Idusogie et al., *J. Immunology*, 164 (8): 4178-84 (2000) and Shields et al., *J. Biol. Chem.*, 276: 6591-6604 (2001), and increased anti-inflammatory properties would be desirable characteristics. Applicants herein have discovered that specific modifications of amino acid positions 243 and 264 in the Fc region of an IgG can impart a lack of, or a significant decrease in, effector function irrespective of the presence of sialylation at the terminal glycan positions. Specifically, Applicants have found that modification to residues F243 and V264 in the Fc region to alanine resulted in an antibody with decreased binding to Fcγ receptors and C1q. It is notable that antibodies produced with these Fc region modifications exhibited decreased binding to Fcγ receptors, regardless of whether they were found to have the α 2,6-linked sialic acid form as the terminal glycan.

As such, Applicants have developed a double Fc mutein, F243A/V264A, which will produce Fc-containing polypeptides having the aforesaid desired characteristics. The Examples herein comprise transforming a host cell with a polynucleotide vector encoding a Fc-containing polypeptide comprising mutations at positions 243 and 264 of the Fc region, and culturing the transformed host cell to produce the Fc-containing polypeptide.

## Production of Fc-Containing Polypeptides

The Fc-containing polypeptides of the invention can be made according to any method known in the art suitable for generating polypeptides comprising a Fc region. In one embodiment, the Fc-containing polypeptide is an antibody or an antibody fragment (including, without limitation a polypeptide consisting of or consisting essentially of the Fc region of an antibody). In another embodiment, the Fc-containing polypeptide is an immunoadhesin. Methods of preparing antibody and antibody fragments are well known in the art. Methods of introducing point mutations into a polypeptide, for example site directed mutagenesis, are also well known in the art.

In the Examples disclosed herein, an IgG1 heavy and light chain containing a consensus C<sub>H</sub>2 sequence and the Fc double mutants described herein were expressed in two different glycoengineered *Pichia pastoris* strains. As described in the Examples that follow, the heavy and light chain gene sequences were under the control of a methanol inducible promoter, AOX1, and incorporated a bleomycin (Zeocin) selection marker. This strategy integrates the entire expression cassette into the Trp2 locus by homologous DNA recombination.

Secreted antibodies were captured from the fermentation broth by protein A affinity chromatography followed by a Source 30S cation exchange purification step. Purified antibodies were characterized by SDS-PAGE (FIG. 2) and size exclusion chromatography (SEC) and reverse phase HPLC to assess proper assembly. As seen in FIG. 2, the antibodies produced by the materials and methods herein had a purity profile on SDS-PAGE that was similar to that for a mammalian cell (CHO) produced Her2 antibody. IgG analysis by SEC and reverse phase HPLC further demonstrated that antibodies made from the Fc mutein glycoengineered strains were properly assembled and were similar to the mammalian cell produced antibody in terms of assembly (data not shown). Antigen affinity for the various antibodies made by the materials and methods herein was determined by a cell based assay using a SK-BR3 cell line, which in this instance was a Her2-overexpressing human breast cancer line. As

expected, all of the antibodies, including the Fc muteins, bound equally well to the SK-BR3 cell line (FIG. 3).

## N-Glycan Analysis of Fc Muteins

For many glycoproteins, including certain antibodies, sialylation of the terminal N-linked glycan of an IgG Fc region is essential for producing glycoproteins and antibodies that have the correct conformation to impart therapeutic activity. See, for example, Anthony et al., *Science*, 320: 373-376 (2008), where terminal sialylation was correlated to anti-inflammatory activity for an IVIG preparation. Sialylation requires the presence of a penultimate galactose, upon which the sialyl transferase acts to form the sialylated glycan. Thus, glycoproteins lacking one or more terminal galactose glycoforms cannot produce antibodies having the α 2,6-linked sialic acid composition associated with anti-inflammatory activity.

Typically, antibodies produced in mammalian cell culture, such as CHO cells, have a glycoform composition comprising: G0F (37%), G1F (43%), G2F (9%), G0 (4%), G1 (3%) and Man5 (3%), which have little or no terminal galactose to act as a substrate for the transfer of sialic acid. In the case of CHO cell production, the terminal glycan produced is the α-2,3-linked form; CHO cells do not express an α-2,6 sialyl transferase necessary to produce the α2,6-linked form of sialic acid, which has been associated with anti-inflammatory activity. However, overexpression of a specific α-2,6 sialyltransferase in CHO can give rise to a mixture of α-2,3-linked and α-2,6-linked sialic acid (Bragonzi et al., *BBA* 1474:273-282 (2000); *Biochem. Biophys. Res. Comm.* 289: 243-249 (2001)). Glycoengineered *Pichia pastoris* GFI5.0 strains, which are capable of producing high levels of galactosylated non-antibody proteins, such as erythropoietin (Hamilton et al., *Science*, 313: 1441-1443 (2006)), produce antibodies with relatively low amounts of a terminal galactose that can be acted upon to form the α 2,6-linked sialylated form (FIG. 4). Antibodies produced in such *Pichia pastoris* strains typically have a composition including glycoforms G0 (60%), G1 (17%), G2 (4%) and Man5 (8%). Even antibodies produced in *Pichia pastoris* GFI6.0 strains, which have a glycan composition comprising G0 (43.5%), G1 (20.8%), G2(2.7%), NANAGGalGlcNAcMan<sub>5</sub>GlcNAc<sub>2</sub> (5.5%), and NANAGal<sub>2</sub>GlcNAc<sub>2</sub>Man<sub>3</sub>GlcNAc<sub>2</sub> (4.9%), have relatively low levels of the α 2,6-linked sialylated form. Thus, antibodies produced in GFI 5.0 and 6.0 strains have much lower levels of galactosylation and sialylation compared to non-antibody proteins (such as erythropoietin) produced in the same strains.

The N-glycan composition of the Her2 antibody and corresponding Fc mutein antibodies produced herein in glycoengineered *Pichia pastoris* GFI5.0 and GFI6.0 strains were analyzed by matrix-assisted laser desorption ionization/time-of-flight (MALDI-TOF) mass spectrometry after release from the antibody with peptide-N-glycosidase F (FIGS. 4-8). Released carbohydrate composition of the Her2 and the Fc mutein antibodies was quantitated by HPLC on an Allentech Prevail carbo (Alltech Associates, Deerfield Ill.) column.

Glycoforms of the *Pichia pastoris* Her2 antibody from the GFI5.0 strain included biantennary G0, G1, and G2 and other neutral glycans, with G0 as the dominant glycoform (FIG. 4). This glycan composition is consistent with that produced commercially in CHO cells, with the exception that the *Pichia pastoris* derived antibody inherently lacks fucose. Conversely, the glycoforms from the Fc mutein antibodies produced in GFI5.0 have dissimilar glycan compositions. The single Fc mutein antibodies, either F243A or V264A, exhibited an increase in galactosylated N-glycans, which can serve as the substrate for the α 2,6 sialyl transferase, as seen from

the highest peak on MALDI-TOF mass spectrometry (FIGS. 5 and 6), while the N-glycan composition for the Fc double mutein antibody, F243A/V264A, exhibited an even greater increase with over 80% of the total N-glycans galactosylated (FIG. 7). The level of G2 (bi-galactosylated N-glycan) present on the Fc double mutein antibody represented the greatest proportion of G2 seen for any antibody evaluated by Applicants. When these same Fc mutein antibodies were expressed in the GFI6.0 strain,  $\alpha$  2,6-linked sialic acid was added to G1 (mono-galactosylated) or G2 (bi-galactosylated) N-glycans. For the single Fc mutein antibody, nearly all of the G2 N-glycan had been converted to a sialylated glycan (data not shown). While antibodies produced from the single Fc muteins exhibited a very high level of  $\alpha$  2,6-linked sialylation (40-51%, see Table 3), the level was less than that for the antibodies produced from the Fc double mutein (FIG. 8 and Table 3) where 74% sialylation was achieved with bioreactor fermentation and 91% or greater sialylation was achieved in small scale fermentation.

Without wishing to be bound by any theory, Applicants believe that the sialic acid transfer to the Fc oligosaccharide is enhanced by the more open pocket configuration of the  $C_H2$  domain imparted by the Fc double mutein as compared to the Fc single mutein. It should also be noted that the  $\alpha$  2,6 form of sialic acid produced from a GFI6.0 strain is the same form produced in humans and differs from the  $\alpha$  2,3-linked sialic acid form present on antibodies produced in CHO cell lines, Jassal et al., *Biochem. Biophys. Res. Comm.* 289: 243-249, 2001.

#### Fc $\gamma$ R Binding of Fc Muteins

Using an ELISA based assay, Applicants compared Fc gamma receptor (Fc $\gamma$ R) binding for the *Pichia pastoris* Her2 antibody, single and double Fc mutein antibodies and the Her2 antibody. As shown in the experiments described in Examples 11 and 15, the Fc double mutein had a decrease in affinity to Fc $\gamma$ RI. As Fc $\gamma$ RI is a receptor shown to stimulate an immune response upon antibody binding, these data suggest that double mutein antibodies will be less capable of promoting an immune response.

For Fc $\gamma$ RIIb, a receptor that has lower antibody affinity as compared to Fc $\gamma$ RI and has been shown to inhibit an immune response, the double Fc mutein binds with reduced affinity. For Fc $\gamma$ RIIa, the double Fc mutein also appears to bind with reduced affinity. These data suggest that the conformational structure of the double mutein antibodies has been altered such that the ability to inhibit an immune response via Fc $\gamma$ RIIa and Fc $\gamma$ IIB/c has been significantly decreased or eliminated.

Fc $\gamma$ RIIIa-F158 and Fc $\gamma$ RIIIa-V158 are polymorphisms of a receptor known to stimulate an immune response, but have lower antibody affinity as compared to Fc $\gamma$ RI. The Fc double mutein had little affinity for Fc $\gamma$ RIIIa-F158 while still retaining some affinity for Fc $\gamma$ RIIIa-V158. Taken together, these data suggest that the double Fc mutein is less prone to activating and recruiting immune cells such as macrophages, monocytes and natural killer cells as compared to parent antibody.

#### C1q Binding of Fc Muteins

Antibody-C1q binding is an important parameter for complement dependant cytotoxicity. Binding activity (affinity) of an immunoglobulin (IgG) molecule to C1q may be determined by a cell based assay such as the one provided herein in Example 13. Those of ordinary skill in the art would recognize and appreciate that the disclosed assay can be easily adapted for use with any IgG molecule.

#### ADCC Effects on Fc Muteins

It is well established that the Fc $\gamma$ RIIIa (CD16) receptor is responsible for antibody-dependent cell-mediated cytotoxicity (ADCC) (Daeron et al., *Annu. Rev. Immunol.* 15: 203-234, 1997). Applicants have found that antibodies produced from the double Fc mutein described herein have decreased Fc $\gamma$ RIIIa binding (FIGS. 9C and D; Tables 4 and 5) and, as such, have likely lost the ability to effect Fc $\gamma$ RIIIa-mediated ADCC.

Example 13 provides an in vitro assay for measuring B-cell depletion and fluorescence released ADCC.

#### Bioavailability of Fc Muteins

Bioavailability refers to the extent to and rate at which an active moiety, whether it be a drug or metabolite, enters human circulation and thereby accessing the site of action. Bioavailability of a drug is mainly affected by the properties of the dosage form as opposed to the drug's physiochemical properties, which in turn can determine its absorption potential. Chemical equivalence suggests that the drug products contain the same active ingredient(s) and in the same amount, although other inactives may differ. Similarly, bioequivalence suggests that the two drug products, when given to the same patient in the same dosing regimen, will produce equivalent concentrations of drug in circulation and tissues. Conversely, two drug products that are not identical may be capable of producing the same therapeutic effect (and adverse effects) when given to the same patient. Thus, the bioavailability of a drug is relative to a determination, of any equivalence in that it may be possible to achieve therapeutic equivalence even when bioavailabilities differ. For drugs having a narrow therapeutic index, i.e. the ratio of the minimum toxic concentration to the median effective concentration, bioavailability differences may result in therapeutic nonequivalence.

Applicants have surprisingly found that the Fc double mutein antibodies produced in *Pichia pastoris* by the materials and methods herein, when injected subcutaneously, had better bioavailability (absorption or exposure) than a comparable antibody produced in CHO cells by known commercial methods. In general, subcutaneous administration is preferable versus intravenous administration in that a subcutaneous formulation may be self-administered by the patient. As shown in FIG. 12, the serum concentrations from mice treated (as set forth in Example 14) with an Fc double mutein antibody increased about 30% as compared to serum concentrations for a CHO produced counterpart and was greater than that for an antibody produced in *Pichia pastoris* lacking the increased  $\alpha$  2,6-linked sialylated form.

#### Production of Human Glycosylated Antibodies Having Increased Levels of Sialylation

As a result of the findings herein, Applicants have developed a method of producing antibodies in that have increased  $\alpha$  2,6-linked sialylation through the modification of the Fc region of the antibody such that a strain is created that can produce in vivo antibodies that have increased galactosylation and sialylation at position Asn297. Without wishing to be bound by any theory, Applicants propose that the lack of sialic acid and the low level of galactose present on the N-glycan at position Asn 297 of an antibody is due, not to a low efficiency of the respective glycosyl transferase, but rather to the steric hindrance inherent in the structure of the Fc region. Applicants believe that the structure of the Fc region prevents the addition of galactose and sialic acid during the short period of time when the antibody passes through the secretory pathway. Previous reports have suggested that high levels of galactosylation of a CD20 mAb was possible in vitro when longer incubation times than would typically be used in vivo are used in conjunction with galactosyltransferase Li et al., *Nat. Biotechnol.* 24(2): 210-215 (2006). It was thought that galactose

transfer occurred when the antibody was in an open configuration with less steric hindrance. Structural or conformational changes resulting from the double mutation, i.e. double Fc mutein, result in a permanently open conformation allowing for greatly increased galactose and sialic acid transfer.

From Applicants studies herein, it appears that the individual mutation of amino acid F243 or V264 had a similar impact on increasing galactosylation and sialylation of the Fc region of an IgG1 molecule. Lund et al., *J. Immunology* 157(11): 4963-4969 (1996) reported a moderate increase of sialylated IgG3 produced in a CHO-K1 cell line by altering single amino acids in the Fc region, Phe241, Phe243, Val264, Aps265 or Tyr296 resulting in levels of 12-42% monosialylated and 4-31% bisialylated form. As reported herein, the antibodies produced by the materials and methods herein had a significant increase in sialylation when the specific sites, F243 or V264, were altered to alanine. Moreover,  $\alpha$  2,6-sialylated species levels in excess of 91% were achieved when both sites were simultaneously mutated (Table 3).

#### Biological Targets

It should be noted that while, in the examples that follow, Applicants exemplify the materials and methods of the invention using IgG1 antibodies having sequences similar to those for commercially available anti-Her2 and anti-TNF antibodies, the invention is not limited to the disclosed antibodies. Those of ordinary skill in the art would recognize and appreciate that the materials and methods herein could be used to produce any Fc-containing polypeptide for which the characteristics of enhanced anti-inflammatory activity or decreased effector function would be desirable. It should further be noted that there is no restriction as to the type of Fc-containing polypeptide or antibody so produced by the invention. The Fc region of the Fc-containing polypeptide could be from an IgA, IgD, IgE, IgG or IgM. In one embodiment, the Fc region of the Fc-containing polypeptide is from an IgG, including IgG1, IgG2, IgG3 or IgG4. In one embodiment, Fc region of the Fc-containing polypeptide is from an IgG1. In specific embodiments the antibodies or antibody fragments produced by the materials and methods herein can be humanized, chimeric or human antibodies.

In some embodiments, the Fc-containing polypeptides of the invention will bind to a biological target that is involved in inflammation.

In some embodiments, the Fc-containing polypeptide of the invention will bind to a pro-inflammatory cytokine. In some embodiments, the Fc-containing polypeptide of the invention will bind to a molecule selected from the group consisting of: TNF- $\alpha$ , IL-1, IL-2, IL-4, IL-5, IL-6, IL-8, IL-9, IL-10, IL-12, IL-15, IL-17, IL-18, IL-20, IL-21, IL-22, IL-23, IL-23R, IL-25, IL-27, IL-33, CD2, CD4, CD11A, CD14, CD18, CD19, CD23, CD25, CD40, CD40L, CD20, CD52, CD64, CD80, CD147, CD200, CD200R, TSLP, TSLPR, PD-1, PDL1, CTLA4, VLA-4, VEGF, PCSK9,  $\alpha$ 4 $\beta$ 7-integrin, E-selectin, Fact II, ICAM-3, beta2-integrin, IFN $\gamma$ , C5, CBL, LCAT, CR3, MDL-1, GITR, ADDL, CGRP, TRKA, IGFI1R, RANKL, GTC,  $\alpha$ BLys, or the receptor for any of the above mentioned molecules. In one embodiment, the Fc-containing polypeptide of the invention will bind to TNF- $\alpha$ . In another embodiment, the Fc-containing polypeptide of the invention will bind to Her2. In another embodiment, the Fc-containing polypeptide of the invention will bind to PCSK9. In another embodiment, the Fc-containing polypeptide of the invention will bind to TNFR. In another embodiment, the Fc-containing polypeptide of the invention will bind to LCAT. In another embodiment, the Fc-containing polypeptide of the invention will bind to TSLP. In another embodiment, the Fc-containing polypeptide of the invention will

bind to PD-1. In another embodiment, the Fc-containing polypeptide of the invention will bind to IL-23.

In some embodiments, the Fc-containing polypeptides of the invention will be specific for an antigen selected from autoimmune antigens, allergens, MHC molecules or Rhesus factor D antigen. See, e.g., the antigens listed in Table 1 of WO2010/10910, which is incorporated herein by reference. Methods of Increasing Anti-Inflammatory Properties or Decreasing Effector Function/Cytotoxicity

The invention also comprises a method of increasing the anti-inflammatory properties of an Fc-containing polypeptide comprising: selecting a parent Fc-containing polypeptide that is useful in treating an inflammatory condition (for example, an antibody or immunoadhesin that binds to an antigen that is involved in inflammation) and introducing mutations at positions 243 and 264 of the Fc-containing polypeptide, wherein the numbering is according to the EU index as in Kabat, wherein the Fc-containing polypeptide has increased anti-inflammatory properties when compared to the parent Fc-containing polypeptide. In a embodiment, the Fc-containing polypeptide comprises mutations F243A and V264A. In another embodiment, the Fc-containing polypeptide comprises mutations F243Y and V264G. In another embodiment, the Fc-containing polypeptide comprises mutations F243T and V264G. In another embodiment, the Fc-containing polypeptide comprises mutations F243L and V264A. In another embodiment, the Fc-containing polypeptide comprises mutations F243L and V264N. In another embodiment, the Fc-containing polypeptide comprises mutations F243V and V264G. In one embodiment, the parent Fc-containing polypeptide is an antibody, antibody fragment or immunoadhesin that binds to an antigen that is involved in inflammation. In one embodiment, the parent Fc-containing polypeptide is an antibody, antibody fragment or immunoadhesin that is already marketed or under development for the treatment of an inflammatory conditions. In another embodiment, the parent Fc-containing polypeptide is an antibody selected from the group consisting of: Muromonab-CD3 (anti-CD3 receptor antibody), Abciximab (anti-CD41 7E3 antibody), Rituximab (anti-CD20 antibody), Daclizumab (anti-CD25 antibody), Basiliximab (anti-CD25 antibody), Palivizumab (anti-RSV (respiratory syncytial virus) antibody), Infliximab (anti-TNF $\alpha$  antibody), Trastuzumab (anti-Her2 antibody), Gemtuzumab ozogamicin (anti-CD33 antibody), Alemtuzumab (anti-CD52 antibody), Ibritumomab tiuxeten (anti-CD20 antibody), Adalimumab (anti-TNF $\alpha$  antibody), Omalizumab (anti-IgE antibody), Tositumomab-131I (iodinated derivative of an anti-CD20 antibody), Efalizumab (anti-CD11a antibody), Cetuximab (anti-EGF receptor antibody), Golimumab (anti-TNF $\alpha$  antibody), Bevacizumab (anti VEGF-A antibody), Natalizumab (anti  $\alpha$ 4 integrin), Efalizumab (anti CD11a), Cetolizumab (anti-TNF $\alpha$  antibody), Tocilizumab (anti-IL-6R), Ustenkinumab (anti IL-12/23), alemtuzumab (anti CD52), and natalizumab (anti  $\alpha$ 4 integrin), and variants thereof. In another embodiment, the parent Fc-containing polypeptide is an Fc-fusion protein selected from the group consisting of Arcalyst/rilonacept (IL1R-Fc fusion), Orencia/abatacept (CTLA-4-Fc fusion), Amevive/alefacept (LFA-3-Fc fusion), Anakinra-Fc fusion (IL-1Ra-Fc fusion protein), etanercept (TNFR-Fc fusion protein), FGF-21-Fc fusion protein, GLP-1-Fc fusion protein, RAGE-Fc fusion protein, ActRIIA-Fc fusion protein, ActRIIB-Fc fusion protein, glucagon-Fc fusion protein, oxyntomodulin-Fc-fusion protein, GM-CSF-Fc fusion protein, EPO-Fc fusion protein, Insulin-Fc fusion protein, proinsulin-Fc fusion protein and insulin precursor-Fc fusion protein, and analogs and variants thereof.

The invention also comprises a method of reducing the effector function of an Fc-containing polypeptide, comprising introducing mutations at positions 243 and 264 of a parent Fc-containing polypeptide, wherein said Fc containing polypeptide has decreased effector function when compared to the parent Fc-containing polypeptide, wherein the numbering is according to the EU index as in Kabat. In one embodiment, the Fc-containing polypeptide comprises mutations F243A and V264A. In one embodiment, the Fc-containing polypeptide is an antibody or antigen binding fragment thereof. In one embodiment, the effector function is ADCC. In another embodiment, the effector function is CDC.

The invention also comprises a method of decreasing cytotoxicity of an Fc-containing polypeptide comprising: selecting a parent Fc-containing polypeptide that is useful in treating an inflammatory condition (for example, an antibody or immunoadhesin that binds to an antigen that is involved in inflammation) that binds to an antigen that is involved in inflammation and introducing mutations at positions 243 and 264 of the Fc-containing polypeptide, wherein the numbering is according to the EU index as in Kabat, wherein the Fc-containing polypeptide has decreased cytotoxicity when compared to the parent Fc-containing polypeptide.

In one embodiment, the parent Fc-containing polypeptide comprises a native Fc region. In another embodiment, the parent Fc-containing polypeptide comprises a F243A mutation. In another embodiment, the parent Fc-containing polypeptide comprises a V264A mutation.

#### Methods of Treatment

The invention also comprises a method of treating an inflammatory condition in a subject in need thereof comprising: administering to the subject a therapeutically effective amount of an Fc-containing polypeptide comprising mutations at positions 243 and 264, wherein the numbering is according to the EU index as in Kabat. In one embodiment, the Fc-containing polypeptide comprises mutations F243A and V264A. In another embodiment, the Fc-containing polypeptide comprises mutations F243Y and V264G. In another embodiment, the Fc-containing polypeptide comprises mutations F243T and V264G. In another embodiment, the Fc-containing polypeptide comprises mutations F243L and V264A. In another embodiment, the Fc-containing polypeptide comprises mutations F243L and V264N. In another embodiment, the Fc-containing polypeptide comprises mutations F243V and V264G. The Fc-containing polypeptide of the invention can be administered by any route. In one embodiment, the Fc-containing polypeptide is administered parenterally. In one embodiment, the Fc-containing polypeptide is administered subcutaneously.

In one embodiment, the inflammatory condition is unwanted inflammatory immune reactions.

In one embodiment, the inflammatory condition is an autoimmune disease. In one embodiment, the inflammatory condition will be multiple sclerosis. In one embodiment, the inflammatory condition is systemic lupus erythematosus. In one embodiment, the inflammatory condition is type I diabetes.

In one embodiment, the inflammatory condition is a primary immunodeficiency syndrome, including congenital agammaglobulinaemia and hypogammaglobulinaemia, common variable immunodeficiency, severe combined immunodeficiency, or Wiskott Aldrich syndrome.

In one embodiment, the inflammatory condition is a secondary immunodeficiency syndrome, including B-cell lymphocytic leukemia, HIV infection or an allogeneic bone marrow transplantation.

In one embodiment, the inflammatory condition is idiopathic thrombocytopenic purpura.

In one embodiment, the inflammatory condition is multiple myeloma.

In one embodiment, the inflammatory condition is Guillain-Barre syndrome.

In one embodiment, the inflammatory condition is Kawasaki disease.

In one embodiment, the inflammatory condition is chronic inflammatory demyelinating polyneuropathy (CIDP).

In one embodiment, the inflammatory condition is autoimmune neutropenia.

In one embodiment, the inflammatory condition is hemolytic anemia.

In one embodiment, the inflammatory condition is anti-Factor VIII autoimmune disease.

In one embodiment, the inflammatory condition is multifocal neuropathy.

In one embodiment, the inflammatory condition is systemic vasculitis (ANCA positive).

In one embodiment, the inflammatory condition is polymyositis.

In one embodiment, the inflammatory condition is dermatomyositis.

In one embodiment, the inflammatory condition is antiphospholipid syndrome.

In one embodiment, the inflammatory condition is sepsis syndrome.

In one embodiment, the inflammatory condition is graft-versus-host disease.

In one embodiment, the inflammatory condition is allergy.

In one embodiment, the inflammatory condition is an anti-Rhesus factor D reaction.

In one embodiment, the inflammatory condition is an inflammatory condition of the cardiovascular system. The Fc-containing polypeptides of the invention may be used to treat atherosclerosis, atherothrombosis, coronary artery hypertension, acute coronary syndrome and heart failure, all of which are associated with inflammation.

In one embodiment, the inflammatory condition is an inflammatory condition of the central nervous system. In another embodiment, the inflammatory condition will be an inflammatory condition of the peripheral nervous system. For example, the Fc-containing polypeptides of the invention may be used for the treatment of, e.g., Alzheimer's disease, amyotrophic lateral sclerosis (a.k.a. ALS; Lou Gehrig's disease), ischemic brain injury, prion diseases, and HIV-associated dementia.

In one embodiment, the inflammatory condition is an inflammatory condition of the gastrointestinal tract. For example, the Fc-containing polypeptides of the invention may be used for treating inflammatory bowel disorders, e.g., Crohn's disease, ulcerative colitis, celiac disease, and irritable bowel syndrome.

In one embodiment, the inflammatory condition is psoriasis, atopic dermatitis, arthritis, including rheumatoid arthritis, osteoarthritis, and psoriatic arthritis.

In one embodiment, the inflammatory condition is steroid-dependent atopic dermatitis.

In one embodiment, the inflammatory condition is cachexia.

Examples of other inflammatory disorders that can be treated using the Fc-containing polypeptides of the invention also include: acne vulgaris, asthma, autoimmune diseases, chronic prostatitis, glomerulonephritis, hypersensitivities,

pelvic inflammatory disease, reperfusion injury, sarcoidosis, transplant rejection, vasculitis, interstitial cystitis and myopathies.

In one embodiment, the Fc-containing polypeptide of the invention will be administered a dose of between 1 to 100 milligrams per kilograms of body weight. In one embodiment, the Fc-containing polypeptide of the invention will be administered a dose of between 0.001 to 10 milligrams per kilograms of body weight. In one embodiment, the Fc-containing polypeptide of the invention will be administered a dose of between 0.001 to 0.1 milligrams per kilograms of body weight. In one embodiment, the Fc-containing polypeptide of the invention will be administered a dose of between 0.001 to 0.01 milligrams per kilograms of body weight.

#### Pharmaceutical Formulations

The invention also comprises pharmaceutical formulations comprising an Fc-containing polypeptide of the invention and a pharmaceutically acceptable carrier.

In one embodiment, the invention relates a pharmaceutical composition comprising an Fc-containing polypeptide, wherein at least 70% of the N-glycans on the Fc-containing polypeptide comprise an oligosaccharide structure selected from the group consisting of  $\text{NANA}_{(1-4)}\text{Gal}_{(1-4)}\text{GlcNAc}_{(2-4)}\text{Man}_3\text{GlcNAc}_2$ , wherein the Fc-containing polypeptide comprises mutations at amino acid positions 243 and 264 of the Fc region, wherein the numbering is according to the EU index as in Kabat. In one embodiment, the mutations are F243A and V264A. In one embodiment, at least 47 mole % of the N-glycans have the structure  $\text{NANA}_2\text{Gal}_2\text{GlcNAc}_2\text{Man}_3\text{GlcNAc}_2$ . In one embodiment, the sialic acid residues in the sialylated N-glycans are attached via an  $\alpha$ -2,6 linkage. In one embodiment, the sialic acid residues in the sialylated N-glycans are attached via an  $\alpha$ -2,6 linkage and there is no detectable level of an  $\alpha$ -2,3 linked sialic acid. In one embodiment, the sialylated N-glycans will comprise no N-glycolylneuraminic acid (NGNA).

As utilized herein, the term "pharmaceutically acceptable" means a non-toxic material that does not interfere with the effectiveness of the biological activity of the active ingredient(s), approved by a regulatory agency of the Federal or a state government or listed in the U.S. Pharmacopoeia or other generally recognized pharmacopoeia for use in animals and, more particularly, in humans. The term "carrier" refers to a diluent, adjuvant, excipient, or vehicle with which the therapeutic is administered and includes, but is not limited to such sterile liquids as water and oils. The characteristics of the carrier will depend on the route of administration.

Pharmaceutical Formulations of therapeutic and diagnostic agents may be prepared by mixing with acceptable carriers, excipients, or stabilizers in the form of, e.g., lyophilized powders, slurries, aqueous solutions or suspensions (see, e.g., Hardman et al. (2001) *Goodman and Gilman's The Pharmacological Basis of Therapeutics*, McGraw-Hill, New York, N.Y.; Gemara (2000) *Remington: The Science and Practice of Pharmacy*, Lippincott, Williams, and Wilkins, New York, N.Y.; Avis, et al. (eds.) (1993) *Pharmaceutical Dosage Forms: Parenteral Medications*, Marcel Dekker, NY; Lieberman, et al. (eds.) (1990) *Pharmaceutical Dosage Forms: Tablets*, Marcel Dekker, NY; Lieberman, et al. (eds.) (1990) *Pharmaceutical Dosage Forms: Disperse Systems*, Marcel Dekker, NY; Weiner and Kotkoskie (2000) *Excipient Toxicity and Safety*, Marcel Dekker, Inc., New York, N.Y.).

The mode of administration can vary. Suitable routes of administration include oral, rectal, transmucosal, intestinal, parenteral; intramuscular, subcutaneous, intradermal, intramedullary, intrathecal, direct intraventricular, intrave-

nous, intraperitoneal, intranasal, intraocular, inhalation, insufflation, topical, cutaneous, transdermal, or intra-arterial.

In certain embodiments, the Fc-containing polypeptides of the invention can be administered by an invasive route such as by injection (see above). In some embodiments of the invention, the Fc-containing polypeptides of the invention, or pharmaceutical composition thereof, is administered intravenously, subcutaneously, intramuscularly, intraarterially, intraarticularly (e.g. in arthritis joints), intratumorally, or by inhalation, aerosol delivery. Administration by non-invasive routes (e.g., orally; for example, in a pill, capsule or tablet) is also within the scope of the present invention.

In certain embodiments, the Fc-containing polypeptides of the invention can be administered by an invasive route such as by injection (see above). In some embodiments of the invention, the Fc-containing polypeptides of the invention, or pharmaceutical composition thereof, is administered intravenously, subcutaneously, intramuscularly, intraarterially, intraarticularly (e.g. in arthritis joints), intratumorally, or by inhalation, aerosol delivery. Administration by non-invasive routes (e.g., orally; for example, in a pill, capsule or tablet) is also within the scope of the present invention.

Compositions can be administered with medical devices known in the art. For example, a pharmaceutical composition of the invention can be administered by injection with a hypodermic needle, including, e.g., a prefilled syringe or autoinjector.

The pharmaceutical compositions of the invention may also be administered with a needleless hypodermic injection device; such as the devices disclosed in U.S. Pat. Nos. 6,620,135; 6,096,002; 5,399,163; 5,383,851; 5,312,335; 5,064,413; 4,941,880; 4,790,824 or 4,596,556.

The pharmaceutical compositions of the invention may also be administered by infusion. Examples of well-known implants and modules for administering pharmaceutical compositions include: U.S. Pat. No. 4,487,603, which discloses an implantable micro-infusion pump for dispensing medication at a controlled rate; U.S. Pat. No. 4,447,233, which discloses a medication infusion pump for delivering medication at a precise infusion rate; U.S. Pat. No. 4,447,224, which discloses a variable flow implantable infusion apparatus for continuous drug delivery; U.S. Pat. No. 4,439,196, which discloses an osmotic drug delivery system having multi-chamber compartments. Many other such implants, delivery systems, and modules are well known to those skilled in the art.

Alternately, one may administer the antibody in a local rather than systemic manner, for example, via injection of the antibody directly into an arthritic joint, often in a depot or sustained release formulation. Furthermore, one may administer the antibody in a targeted drug delivery system, for example, in a liposome coated with a tissue-specific antibody, targeting, for example, arthritic joint or pathogen-induced lesion characterized by immunopathology. The liposomes will be targeted to and taken up selectively by the afflicted tissue.

The administration regimen depends on several factors, including the serum or tissue turnover rate of the therapeutic antibody, the level of symptoms, the immunogenicity of the therapeutic antibody, and the accessibility of the target cells in the biological matrix. Preferably, the administration regimen delivers sufficient therapeutic antibody to effect improvement in the target disease state, while simultaneously minimizing undesired side effects. Accordingly, the amount of biologic delivered depends in part on the particular therapeutic antibody and the severity of the condition being treated. Guidance in selecting appropriate doses of therapeu-

tic antibodies is available (see, e.g., Wawrzynczak (1996) *Antibody Therapy*, Bios Scientific Pub. Ltd, Oxfordshire, UK; Kresina (ed.) (1991) *Monoclonal Antibodies, Cytokines and Arthritis*, Marcel Dekker, New York, N.Y.; Bach (ed.) (1993) *Monoclonal Antibodies and Peptide Therapy in Autoimmune Diseases*, Marcel Dekker, New York, N.Y.; Baert, et al. (2003) *New Engl. J. Med.* 348:601-608; Milgrom et al. (1999) *New Engl. J. Med.* 341:1966-1973; Slamon et al. (2001) *New Engl. J. Med.* 344:783-792; Beniaminovitz et al. (2000) *New Engl. J. Med.* 342:613-619; Ghosh et al. (2003) *New Engl. J. Med.* 348:24-32; Lipsky et al. (2000) *New Engl. J. Med.* 343:1594-1602).

Determination of the appropriate dose is made by the clinician, e.g., using parameters or factors known or suspected in the art to affect treatment. Generally, the dose begins with an amount somewhat less than the optimum dose and it is increased by small increments thereafter until the desired or optimum effect is achieved relative to any negative side effects. Important diagnostic measures include those of symptoms of, e.g., the inflammation or level of inflammatory cytokines produced. Preferably, a biologic that will be used is derived from the same species as the animal targeted for treatment, thereby minimizing any immune response to the reagent. In the case of human subjects, for example, chimeric, humanized and fully human Fc-containing polypeptides are preferred.

Fc-containing polypeptides can be provided by continuous infusion, or by doses administered, e.g., daily, 1-7 times per week, weekly, bi-weekly, monthly, bimonthly, quarterly, semiannually, annually etc. Doses may be provided, e.g., intravenously, subcutaneously, topically, orally, nasally, rectally, intramuscular, intracerebrally, intraspinally, or by inhalation. A total weekly dose is generally at least 0.05  $\mu\text{g}/\text{kg}$  body weight, more generally at least 0.2  $\mu\text{g}/\text{kg}$ , 0.5  $\mu\text{g}/\text{kg}$ , 1  $\mu\text{g}/\text{kg}$ , 10  $\mu\text{g}/\text{kg}$ , 100  $\mu\text{g}/\text{kg}$ , 0.25 mg/kg, 1.0 mg/kg, 2.0 mg/kg, 5.0 mg/ml, 10 mg/kg, 25 mg/kg, 50 mg/kg or more (see, e.g., Yang et al., *New Engl. J. Med.* 349:427-434 (2003); Herold et al., *New Engl. J. Med.* 346:1692-1698 (2002); Liu et al., *J. Neurol. Neurosurg. Psych.* 67:451-456 (1999); Portielji et al., *Cancer Immunol. Immunother.* 52:133-144 (2003). In other embodiments, an Fc-containing polypeptide of the present invention is administered subcutaneously or intravenously, on a weekly, biweekly, "every 4 weeks," monthly, bimonthly, or quarterly basis at 10, 20, 50, 80, 100, 200, 500, 1000 or 2500 mg/subject.

As used herein, the terms "therapeutically effective amount", "therapeutically effective dose" and "effective amount" refer to an amount of an Fc-containing polypeptide of the invention that, when administered alone or in combination with an additional therapeutic agent to a cell, tissue, or subject, is effective to cause a measurable improvement in one or more symptoms of a disease or condition or the progression of such disease or condition. A therapeutically effective dose further refers to that amount of the Fc-containing polypeptide sufficient to result in at least partial amelioration of symptoms, e.g., treatment, healing, prevention or amelioration of the relevant medical condition, or an increase in rate of treatment, healing, prevention or amelioration of such conditions. When applied to an individual active ingredient administered alone, a therapeutically effective dose refers to that ingredient alone. When applied to a combination, a therapeutically effective dose refers to combined amounts of the active ingredients that result in the therapeutic effect, whether administered in combination, serially or simultaneously. An effective amount of a therapeutic will result in an improvement of a diagnostic measure or parameter by at least 10%; usually by at least 20%; preferably at least about 30%; more

preferably at least 40%, and most preferably by at least 50%. An effective amount can also result in an improvement in a subjective measure in cases where subjective measures are used to assess disease severity.

#### EXAMPLE 1

##### Strains and Reagents

*Escherichia coli* strains TOP10 or DH5 $\alpha$  (Invitrogen, CA) were used for recombinant DNA work. Restriction endonucleases, DNA modification enzymes and PNGase F were obtained from New England Biolabs, Ipswich, Mass. Oligonucleotides were ordered from Integrated DNA Technologies, Coralville, Iowa.

#### EXAMPLE 2

##### Construction of Anti-Her 2 IgG1 Fc Muteins and *Pichia pastoris* Recombinant Expression Vector

The preparation of single and double Fc muteins of Her2 IgG1 monoclonal antibody in *Pichia pastoris* was carried out using the sequences and protocols listed below.

##### A. Heavy and Light Chains

The heavy and light chain sequences, SEQ ID NOS: 1 and 2, respectively, used for the preparation of the Her2 monoclonal IgG1 antibody are as set forth below. The amino acid sequence of the heavy chain anti-Her2 double mutein antibody is shown in SEQ ID NO:9. The heavy and light chains were codon optimized according to *Pichia pastoris* codon usage and synthesized by GeneArt AG (Josef-Engert-Str. 11, D-93053 Regensburg, Germany) and cloned into pUC19.

The alanine Fc mutations at amino acid position F243 were carried out using a QuikChange® Site-Directed Mutagenesis Kit (Stratagene, CA) using the forward and reverse primers, FcF243A-F (SEQ ID NO: 3) and FcF243A-R (SEQ ID NO: 4), respectively. Similarly, mutations at amino acid position V264 were carried out using the forward and reverse primers, V254A-F (SEQ ID NO: 5) and V264A-R (SEQ ID NO: 6), respectively. The double mutation was carried out through the use of enzymatic digestion between the F243A and V264A sites from each of the single mutein plasmids followed by ligation.

##### B. Signal Sequence

The signal sequence of an  $\alpha$ -Mating Factor predomain was fused in frame to the 5' end of the light or heavy chain by PCR fusion. The sequence was codon optimized as described above. A Kozak sequence AAACG was added to the 5' end of the methionine and an EcoR1 site was added before the Kozak sequence for cloning purposes. The DNA sequence (SEQ ID NO: 7) and amino acid (SEQ ID NO: 8) translation are as shown below.

##### C. Recombinant Plasmids for Expression IgG1 and IgG1 Fc Muteins

The heavy and light chains with the fused signal sequence of IgG1 and its muteins were cloned under *Pichia pastoris* AOX1 promoter and in front of *S. cerevisiae* Cyc terminator, respectively. The expression cassette of the completed heavy and light chains was put together into the final expression vector. Genomic insertion into *Pichia pastoris* was achieved by linearization of the vector with Spe1 and targeted integration into the Trp2 site.

A summary of the plasmids used herein is given below in Table 1. A graphic representation of the final expression plasmid for the Her2 double Fc mutein is set forth in FIG. 1.



TABLE 1

Plasmid	Description
pGLY2336	pCR2.1 topo with WT light chain with alpha-MF pre signal sequence and Kozak sequence
pGLY2337	pCR2.1 topo with WT heavy chain with alpha-MF pre signal sequence and Kozak sequence
pGLY2338	WT light chain expression vector
pGLY2987	WT heavy chain expression vector
pGLY2988	WT IgG1 expression final vector having both heavy and light chain
pGLY3067	pCR2.1 topo vector with IgG1 heavy chain F243A mutation
pGLY3473	pCR2.1 topo vector with IgG1 heavy chain V264A mutation
pGLY3474	F243A mutein expression vector
pGLY3475	V264A mutein expression vector
pGLY3479	F243 and V264A double mutein expression vector
pGLY3481	pGLY3474(BmHI/NotI) + pGLY2338 (BglII/Not) F243A mutant with 1 copy Light chain
pGLY3482	pGLY3475(BmHI/NotI) + pGLY2338 (BglII/Not) V264A mutant with 1 copy Light chain
pGLY3483	F243A and V264A double mutations final expression vector with both heavy and light chain

## EXAMPLE 3

Glycoengineered *Pichia* GFI5.0 and GFI6.0 Hosts for Producing Anti-Her2 and its Fc Muteins

Two different glycoengineered *Pichia* hosts were applied in this invention, GFI5.0 and GFI 6.0. Following the procedures disclosed in Gerngross, U.S. Pat. No. 7,029,872 and Gerngross, U.S. Pat. No. 7,449,308, one can construct vectors that are useful for genetically engineering lower eukaryotic host cells such that they are capable of expressing a desired polypeptide having a desired N-glycoform as the predominant species. GFI 5.0 and GFI6.0 strains were engineered from NRRL11430 (American Type Culture Collection (ATCC), P.O. Box 1549, Manassas, Va. 20108, USA) according to the methods described in Hamilton et al., *Science*, 313: 1441-1443 (2006) and Hamilton US 2006/0286637. The engineered *Pichia pastoris* strain GFI5.0 is capable of producing proteins with a biantennary N-glycan structure with terminal galactose. The genotype of the GFI5.0 strain used herein, RDP697, is as follows: *ura5Δ::ScSUC2 och1Δ::lacZ bmt2Δ::lacZ/KIMNN2-2 mnn4L1Δ::lacZ/MmSLC35A3 pno1Δ::lacZ ADE1::lacZ/FB8/NA10/MmSLC35A3 his1::lacZ-URA5-lacZ/XB33/SpGALE/DmUGT arg1::HIS1/KD53/TC54*. The genotype of the engineered *Pichia pastoris* strain GFI 6.0, YGLY3582, is as follows: *ura5Δ::ScSUC2 och1Δ::lacZ, bmt2Δ::lacZ/KIMNN2-2 mnn4L1Δ::lacZ/MmSLC35A3, pno1Δmnn4Δ::lacZ met16Δ::lacZ, his1Δ::lacZ/ScGAL10/XB33/DmUGT, arg1Δ::HIS1/KD53/TC54, ADE1::lacZ/NA10/MmSLC35A3/FB8, PRO1::lacZ-URA5-lacZ/TrMDS1, TRP2:ARG1/MmCST/HsGNE/HsCSS/HsSPS/MmST6-33Y*. The GFI 6.0 strain is capable of producing proteins with a biantennary N-glycan structure on which terminal  $\alpha$  2,6-linked sialic acid is attached to galactose.

The abbreviations used to describe the genotypes are commonly known and understood by those skilled in the art, and include the following abbreviations:

ScSUC2 *S. cerevisiae* Invertase

OCH1 Alpha-1,6-mannosyltransferase

KIMNN2-2 *K. lactis* UDP-GlcNAc transporter

BMT1 Beta-mannose-transfer (beta-mannose elimination)

BMT2 Beta-mannose-transfer (beta-mannose elimination)

BMT3 Beta-mannose-transfer (beta-mannose elimination)

- BMT4 Beta-mannose-transfer (beta-mannose elimination)  
MNN4L1 MNN4-like 1 (charge elimination)  
MmSLC35A3 Mouse homologue of UDP-GlcNAc transporter  
5 PNO1 Phosphomannosylation of N-glycans (charge elimination)  
MNN4 Mannosyltransferase (charge elimination)  
ScGAL10 UDP-glucose 4-epimerase  
XB33 Truncated HsGalT1 fused to ScKRE2 leader  
10 DmUGT UDP-Galactose transporter  
KD53 Truncated DmMNSII fused to ScMNN2 leader  
TC54 Truncated RnGNTII fused to ScMNN2 leader  
NA10 Truncated HsGNTI fused to PpSEC12 leader  
FB8 Truncated MmMNS1A fused to ScSEC12 leader  
15 TrMDS1 Secreted *T. reesei* MNS1  
ADE1 N-succinyl-5-aminoimidazole-4-carboxamide ribotide (SAICAR) synthetase  
MmCST Mouse CMP-sialic acid transporter  
HsGNE Human UDP-GlcNAc 2-epimerase/N-acetylmannosamine kinase  
20 HsCSS Human CMP-sialic acid synthase  
HsSPS Human N-acetylneuraminase-9-phosphate synthase  
MmST6-33 Truncated Mouse alpha-2,6-sialyl transferase fused to ScKRE2 leader  
25 LmSTT3d Catalytic subunit of oligosaccharyltransferase from *Leishmania major*

## EXAMPLE 4

## 30 Yeast Transformation and Screening

The glycoengineered GFI5.0 and GS6.0 strains were grown in YPD rich media (yeast extract 1%, peptone 2% and 2% dextrose), harvested in the logarithmic phase by centrifugation, and washed three times with ice-cold 1 M sorbitol. One to five  $\mu$ g of a SpeI digested plasmid was mixed with competent yeast cells and electroporated using a Bio-Rad Gene Pulser Xcell™ (Bio-Rad, 2000 Alfred Nobel Drive, Hercules, Calif. 94547) preset *Pichia pastoris* electroporation program. After one hour in recovery rich media at 24° C., the cells were plated on a minimal dextrose media (1.34% YNB, 0.0004% biotin, 2% dextrose, 1.5% agar) plate containing 300  $\mu$ g/ml Zeocin and incubated at 24° C. until the transformants appeared.

To screen for high titer strains, 96 transformants were inoculated in buffered glycerol-complex medium (BMGY) and grown for 72 hours followed by a 24 hour induction in buffered methanol-complex medium (BMMY). Secretion of antibody was assessed by a Protein A beads assay as follows. Fifty micro liter supernatant from 96 well plate cultures was diluted 1:1 with 50 mM Tris pH 8.5 in a non-binding 96 well assay plate. For each 96 well plate, 2 ml of magnetic BioMag Protein A suspension beads (Qiagen, Valencia, Calif.) were placed in a tube held in a magnetic rack. After 2-3 minutes when the beads collected to the side of the tube, the buffer was decanted off. The beads were washed three times with a volume of wash buffer equal to the original volume (100 mM Tris, 150 mM NaCl, pH 7.0) and resuspended in the same wash buffer. Twenty  $\mu$ l of beads were added to each well of the assay plate containing diluted samples. The plate was covered, vortexed gently and then incubated at room temperature for 1 hour, while vortexing every 15 minutes. Following incubation, the sample plate was placed on a magnetic plate inducing the beads to collect to one side of each well. On the Biomek NX Liquid Handler (Beckman Coulter, Fullerton, Calif.), the supernatant from the plate was removed to a waste container. The sample plate was then removed from the magnet and the beads were washed with 100  $\mu$ l wash buffer. The

plate was again placed on the magnet before the wash buffer was removed by aspiration. Twenty  $\mu$ l loading buffer (Invitrogen E-PAGE gel loading buffer containing 25 mM NEM (Pierce, Rockford, Ill.)) was added to each well and the plate was vortexed briefly. Following centrifugation at 500 rpm on the Beckman Allegra 6 centrifuge, the samples were incubated at 99° C. for five minutes and then run on an E-PAGE high-throughput pre-cast gel (Invitrogen, Carlsbad, Calif.). Gels were covered with gel staining solution (0.5 g Coomassie G250 Brilliant Blue, 40% MeOH, 7.5% Acetic Acid), heated in a microwave for 35 seconds, and then incubated at room temperature for 30 minutes. The gels were de-stained in distilled water overnight. High titer colonies were selected for further Sixfors fermentation screening described in detail in Example 5. A summary of the IgG1 wild type (parent) and Fc mutein producing strains is given below in Table 2.

TABLE 2

Strains	Genotype	Description
YDX477	ura5 $\Delta$ ::ScSUC2 och1 $\Delta$ ::lacZ bmt2 $\Delta$ ::lacZ/KIMNN2-2 mnn4L1 $\Delta$ ::lacZ/MmSLC35A3 pno1 $\Delta$ ::lacZ ADE1::lacZ/FB8/NA10/MmSLC35A3 his1::lacZ-URA5-lacZ/XB33/SpGALE/DmUGT arg1::HIS1/KD53/TC54	GF15.0 strain producing WT IgG1
YDX551	ura5 $\Delta$ ::ScSUC2 och1 $\Delta$ ::lacZ bmt2 $\Delta$ ::lacZ/KIMNN2-2 mnn4L1 $\Delta$ ::lacZ/MmSLC35A3 pno1 $\Delta$ ::lacZ ADE1::lacZ/FB8/NA10/MmSLC35A3 his1::lacZ-URA5-lacZ/XB33/SpGALE/DmUGT arg1::HIS1/KD53/TC54	GF15.0 strain producing anti-Her2 Fc V264A mutein
YDX552	ura5 $\Delta$ ::ScSUC2 och1 $\Delta$ ::lacZ bmt2 $\Delta$ ::lacZ/KIMNN2-2 mnn4L1 $\Delta$ ::lacZ/MmSLC35A3 pno1 $\Delta$ ::lacZ ADE1::lacZ/FB8/NA10/MmSLC35A3 his1::lacZ-URA5-lacZ/XB33/SpGALE/DmUGT arg1::HIS1/KD53/TC54	GF15.0 strain producing anti-Her2 Fc F243A mutein
YDX557	ura5 $\Delta$ ::ScSUC2 och1 $\Delta$ ::lacZ bmt2 $\Delta$ ::lacZ/KIMNN2-2 mnn4L1 $\Delta$ ::lacZ/MmSLC35A3 pno1 $\Delta$ ::lacZ ADE1::lacZ/FB8/NA10/MmSLC35A3 his1::lacZ-URA5-lacZ/XB33/SpGALE/DmUGT arg1::HIS1/KD53/TC54	GF15.0 strain producing anti-Her2 Fc F243A, V264A double mutein
YGLY4570	ura5 $\Delta$ ::ScSUC2 och1 $\Delta$ ::lacZ, bmt2 $\Delta$ ::lacZ/KIMNN2-2 mnn4L1 $\Delta$ ::lacZ/MmSLC35A3, pno1 $\Delta$ mnn4 $\Delta$ ::lacZ met16 $\Delta$ ::lacZ, his1 $\Delta$ ::lacZ/ScGAL10/XB33/DmUGT, arg1 $\Delta$ ::HIS1/KD53/TC54, ADE1::lacZ/NA10/MmSLC35A3/FB8, PRO1::lacZ-URA5-lacZ/TrMDS1, TRP2:ARG1/MmCST/HsGNE/HsCSS/HsSPS/MmST6-33	GF16.0 strain making anti-Her2 Fc V264A mutein
YGLY4568	ura5 $\Delta$ ::ScSUC2 och1 $\Delta$ ::lacZ, bmt2 $\Delta$ ::lacZ/KIMNN2-2 mnn4L1 $\Delta$ ::lacZ/MmSLC35A3, pno1 $\Delta$ mnn4 $\Delta$ ::lacZ met16 $\Delta$ ::lacZ, his1 $\Delta$ ::lacZ/ScGAL10/XB33/DmUGT, arg1 $\Delta$ ::HIS1/KD53/TC54, ADE1::lacZ/NA10/MmSLC35A3/FB8, PRO1::lacZ-URA5-lacZ/TrMDS1, TRP2:ARG1/MmCST/HsGNE/HsCSS/HsSPS/MmST6-33	GF16.0 strain making anti-Her2 Fc F243A mutein
YGLY4563	ura5 $\Delta$ ::ScSUC2 och1 $\Delta$ ::lacZ, bmt2 $\Delta$ ::lacZ/KIMNN2-2 mnn4L1 $\Delta$ ::lacZ/MmSLC35A3, pno1 $\Delta$ mnn4 $\Delta$ ::lacZ met16 $\Delta$ ::lacZ, his1 $\Delta$ ::lacZ/ScGAL10/XB33/DmUGT, arg1 $\Delta$ ::HIS1/KD53/TC54, ADE1::lacZ/NA10/MmSLC35A3/FB8, PRO1::lacZ-URA5-lacZ/TrMDS1, TRP2:ARG1/MmCST/HsGNE/HsCSS/HsSPS/MmST6-33	GF16.0 strain making anti-Her2 F243A/V264A double mutein

## EXAMPLE 5

## Bioreactor (Sixfors) Screening

Bioreactor fermentation screening was conducted as described as follows: Fed-batch fermentations of glycoengineered *Pichia pastoris* were executed in 0.5 liter bioreactors (Sixfors multi-fermentation system, ATR Biotech, Laurel, Md.) under the following conditions: pH 6.5, 24° C., 300 ml airflow/min, and an initial stirrer speed of 550 rpm with an initial working volume of 350 ml (330 ml BMGY medium [100 mM potassium phosphate, 10 g/l yeast extract, 20 g/l peptone (BD, Franklin Lakes, N.J.), 40 g/l glycerol, 18.2 g/l sorbitol, 13.4 g/l YNB (BD, Franklin Lakes, N.J.), 4 mg/l biotin] and 20 ml inoculum). IRIS multi-fermentor software

(ATR Biotech, Laurel, Md.) was used to increase the stirrer speed from 550 rpm to 1200 rpm linearly between hours 1 and 10 of the fermentation. Consequently, the dissolved oxygen concentration was allowed to fluctuate during the fermentation. The fermentation was executed in batch mode until the initial glycerol charge (40 g/l) was consumed (typically 18-24 hours). A second batch phase was initiated by the addition of 17 ml of a glycerol feed solution to the bioreactor (50% [w/w] glycerol, 5 mg/l biotin and 12.5 ml/l PTM1 salts (65 g/l FeSO<sub>4</sub>·7H<sub>2</sub>O, 20 g/l ZnCl<sub>2</sub>, 9 g/l H<sub>2</sub>SO<sub>4</sub>, 6 g/l CuSO<sub>4</sub>·5H<sub>2</sub>O, 5 g/l H<sub>2</sub>SO<sub>4</sub>, 3 g/l MnSO<sub>4</sub>·7H<sub>2</sub>O, 500 mg/l CoCl<sub>2</sub>·6H<sub>2</sub>O, 200 mg/l NaMoO<sub>4</sub>·2H<sub>2</sub>O, 200 mg/l biotin, 80 mg/l NaI, 20 mg/l H<sub>3</sub>BO<sub>4</sub>). The fermentation was again operated in batch mode until the added glycerol was consumed (typically 6-8 hours). The induction phase was initiated by feeding a methanol solution (100% [w/w] methanol, 5 mg/l biotin and 12.5 ml/l

PTM1 salts) at 0.6 g/hr, typically for 36 hours prior to harvest. The entire volume was removed from the reactor and centrifuged in a Sorvall Evolution RC centrifuge equipped with a SLC-6000 rotor (Thermo Scientific, Milford, Mass.) for 30 minutes at 8,500 rpm. The cell mass was discarded and the supernatant retained for purification and analysis. Glycan quality is assessed by MALDI-Time-of-flight (TOF) spectrometry and 2-aminobenzidine (2-AB) labeling according to Li et al., *Nat. Biotech.* 24(2): 210-215 (2006). Glycans were released from the antibody by treatment with PNGase-F and analyzed by MALDI-TOF to confirm glycan structures. To quantitated the relative amounts of neutral and charged glycans present, the N-glycosidase F released glycans were labeled with 2-AB and analyzed by HPLC.

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## EXAMPLE 6

## Bioreactor Cultivations

Fermentations were carried out in 3L (Applikon, Foster City, Calif.) and 15L (Applikon, Foster City, Calif.) glass bioreactors and a 40L (Applikon, Foster City, Calif.) stainless steel, steam in place bioreactor. Seed cultures were prepared by inoculating BMGY media directly with frozen stock vials at a 1% volumetric ratio. Seed flasks were incubated at 24° C. for 48 hours to obtain an optical density (OD<sub>600</sub>) of 20±5 to ensure that cells are growing exponentially upon transfer. The cultivation medium contained 40 g glycerol, 18.2 g sorbitol, 2.3 g K<sub>2</sub>HPO<sub>4</sub>, 11.9 g KH<sub>2</sub>PO<sub>4</sub>, 10 g yeast extract (BD, Franklin Lakes, N.J.), 20 g peptone (BD, Franklin Lakes, N.J.), 4×10<sup>-3</sup> g biotin and 13.4 g Yeast Nitrogen Base (BD, Franklin Lakes, N.J.) per liter. The bioreactor was inoculated with a 10% volumetric ratio of seed to initial media. Cultivations were done in fed-batch mode under the following conditions: temperature set at 24±0.5° C., pH controlled at to 6.5±0.1 with NH<sub>4</sub>OH, dissolved oxygen was maintained at 1.7±0.1 mg/L by cascading agitation rate on the addition of O<sub>2</sub>. The airflow rate was maintained at 0.7 vvm. After depletion of the initial charge glycerol (40 g/L), a 50% glycerol solution containing 12.5 mL/L of PTM1 salts was fed exponentially at 50% of the maximum growth rate for eight hours until 250 g/L of wet cell weight was reached. Induction was initiated after a thirty minute starvation phase when methanol was fed exponentially to maintain a specific growth rate of 0.01 h<sup>-1</sup>. When an oxygen uptake rate of 150 mM/L/h was reached, the methanol feed rate was kept constant to avoid oxygen limitation.

## EXAMPLE 7

## Antibody Purification

Purification of secreted antibody can be performed by one of ordinary skill in the art using available published methods, for example Li et al., *Nat. Biotech.* 24(2):210-215 (2006), in which antibodies are captured from the fermentation supernatant by Protein A affinity chromatography and further purified using hydrophobic interaction chromatography with a phenyl sepharose fast flow resin.

## EXAMPLE 8

## MALDI-TOF Analysis of Glycans

N-glycans were analyzed as described in Choi et al., *Proc. Natl. Acad. Sci. USA* 100: 5022-5027 (2003) and Hamilton et al., *Science* 301: 1244-1246 (2003). After the glycoproteins were reduced and carboxymethylated, N-glycans were released by treatment with peptide-N-glycosidase F. The released oligosaccharides were recovered after precipitation of the protein with ethanol. Molecular weights were determined by using a Voyager PRO linear MALDI-TOF (Applied Biosystems) mass spectrometer with delayed extraction according to the manufacturer's instructions. The results of the N-glycan analysis of the anti-Her2 antibodies produced according to the Examples above are shown in FIGS. 4-8.

## EXAMPLE 9

## N-Linked Glycan Analysis by HPLC

To quantify the relative amount of each glycoform, the N-glycosidase F released glycans were labeled with 2-aminobenzidine (2-AB) and analyzed by HPLC as described in Choi et al., *Proc. Natl. Acad. Sci. USA* 100: 5022-5027 (2003) and Hamilton et al., *Science* 313: 1441-1443 (2006). The

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amounts of sialylated anti-Her2 antibody produced in a GFI 6.0 strain for single and double muteins produced in 3L bioreactors and for wild-type and double mutein produced in a small scale 0.5L bioreactor is shown in Table 3.

TABLE 3

	Mono-sialylated	Bi-sialylated	Total sialylated
Wild-type	10.4%	0%	10.4%
Single mutein V264A	43%	8%	51%
Single mutein F243A	23%	7%	30%
Double mutein F243A/V264A	27%	47%	74%
Double mutein F243A/V264A (0.5 L bioreactor)	25%	66%	>91%

## EXAMPLE 10

## Antigen Affinity Assay

Mammalian cells expressing antigen were harvested by trypsinization, filtered through a 40 µm cell strainer, and suspended in 1% fetal bovine serum (FBS) in phosphate buffered saline (PBS) in a 96-deep-well plate. Serial dilutions of the purified antibody were added to the cells at final concentration ranging from 10 to 0.01 µg ml<sup>-1</sup>. The antibody cell mixture was incubated for 45 minutes on ice. The cells were washed in cold PBS and stained with 2 µg ml<sup>-1</sup> of anti-human IgG-AlexaFluor488 (Invitrogen, Carlsbad, Calif.) in 1% FBS in PBS for 45 min on ice in the dark. The cells were washed again in cold PBS, suspended in 1% FBS in PBS and transferred to a U-bottom 96-well plate (USA Scientific, Ocala, Fla.). Mean fluorescence intensity (MFI) was detected on a Guava ExpressPlus (Millipore, Billerica, Mass.) using an excitation wavelength of 488 nm and an emission wavelength of 525 nm. The results are shown in FIG. 3.

## EXAMPLE 11

## FcγR Binding Assay

Fcγ receptor binding assays were carried out as described in Shields et al., *J. Biol. Chem.* 276: 6591-6604 (2001) with minor modifications. High protein binding 96-well plates (Corning Costar, Lowell, Mass.) were coated with 100 µl per well of Fcγ receptor solutions in PBS at the following concentrations: 1 µg/mL for FcγRI (R & D Systems) and FcγRIIIa (*Pichia pastoris* produced), 2 µg/mL for FcγRIIb/c (*P. pastoris* produced), 0.4 µg/mL for FcγRIIIa-V158, and 0.8 µg/mL for FcγRIIIa-F158 (both *P. pastoris* produced). FcγRIIIa-V158 and FcγRIIIa-F158 receptors were expressed using *P. pastoris* as described in Li et al., *Nat. Biotech.* 24:210-215 (2006).

FcγRIIIa was also expressed in glycoengineered *Pichia* using a similar method as described in Li et al. The FcγRIIIa extracellular domain was PCR amplified from human cDNA and cloned into pCR2.1 topo vector. The Fc gamma receptors were cloned into *Pichia* expression vector using *S. cerevisiae* alpha Mating Factor prepro domain and under AOX1 promoter. The final strain, yGLY4665, was generated by transforming pGLY3249 into yGLY638 (GFI2.0 host).

The FcγRIIb/c was expressed and produced using glycoengineered *Pichia* YGLY638 (GFI2.0 host). The DNA sequence of the extracellular domain of the human Fc gamma receptor IIb/c (NP\_003992) carrying its C-terminal 9 His-tag was *Pichia* codon optimized, and designated pAS197 (Gene-

Art, Germany). The amino acid sequence of the histidine-tagged extracellular domain of the Fc $\gamma$ RIIb/c can be found in SEQ ID NO:17. For the plasmid construction of pGLY3246, the codon-optimized hFc $\gamma$ RIIb/c (AfeI/KpnI) and *Saccharomyces cerevisiae*  $\alpha$ MFprepro (EcoRI/blunt) were cloned into pGLY2219 at EcoRI and KpnI sites. The resulting plasmid pGLY3246 was transformed into yGLY638 to generate yGLY4653. YGLY4653 were fermented and purified according to Li et al.

For Fc $\gamma$ RI, the antibody was coated in assay diluent (1% BSA, PBS, 0.05% Tween20) in monomeric form. For all other receptors, the antibody was coated after dimerization with alkaline phosphatase conjugated anti-human IgG F(ab')<sub>2</sub> (Jackson ImmunoResearch, West Grove, Pa.) for one hour at room temperature. Fc $\gamma$ RI bound antibody was also detected using the F(ab')<sub>2</sub> and all plates were quantified by measuring excitation at 340 nm and emission at 465 nm after an 18 hour incubation with SuperPhosk (Virolabs, Chantilly, Va.).

The results are shown in FIG. 9. As shown in FIG. 9A, the Fc single muteins ( $\blacktriangle$  and  $\blacktriangledown$ ) had Fc $\gamma$ RI (Fc receptor gamma-chain I, CD64) binding similar to both the Her2 antibody ( $\blacksquare$ ) (FIG. 9A) and the *Pichia pastoris* Her2 (data not shown), while the Fc double mutein ( $\blacklozenge$ ) had about a fourteen fold decrease in affinity to Fc $\gamma$ RI (FIG. 9A).

For Fc $\gamma$ RIIb/c, the Fc single muteins ( $\blacktriangle$  and  $\blacktriangledown$ ) demonstrated a ten fold decrease in receptor binding properties as compared to the Her2 antibody ( $\blacksquare$ ) (FIG. 9B), while the double Fc mutein does not appear to bind to Fc $\gamma$ RIIb/c.

Fc $\gamma$ RIIIa-F158 and Fc $\gamma$ RIIIa-V158, both single Fc muteins ( $\blacktriangle$  and  $\blacktriangledown$ ) bind twenty fold better than the commercial Herceptin antibody ( $\blacksquare$ ) to Fc $\gamma$ RIIIa-F158 (FIG. 9C), but bind Fc $\gamma$ RIIIa-V158 only slightly better than the commercial Herceptin antibody (FIG. 9D). The Fc double mutein ( $\blacklozenge$ ) had little affinity (50 fold decrease) for Fc $\gamma$ RIIIa-F158 (FIG. 9C) while still retaining some affinity for Fc $\gamma$ RIIIa-V158, albeit thirty fold weaker than the commercial Herceptin antibody ( $\blacksquare$ ) and the *Pichia pastoris* Her2 antibody (data not shown) from GS5.0 (FIG. 9D).

Moreover, the affinity of the double Fc mutein for both polymorphisms of Fc $\gamma$ RIIIa does not appear to change upon release of the sialic acid by neuraminidase (data not shown). Thus, without wishing to be bound by any theory, Applicants attribute the decrease in affinity for both polymorphisms of Fc $\gamma$ RIIIa to structural or conformational changes resulting from the double mutation, i.e. double Fc mutein, and not due to the increased levels of the  $\alpha$  2,6-linked sialylated N-glycans.

#### EXAMPLE 12

##### C1q Binding Assay for the Anti-Her2 Antibodies and its Fc Muteins

The C1q binding assay was conducted using the methods of Idusogie et al., *J. Immunology*, 164: 4178-4184 (2000) as described. Serially diluted antibody was coated 100  $\mu$ l per well in 50 mM Na<sub>2</sub>HCO<sub>3</sub> pH9.0 to clear high binding plates. Human C1q complement (US Biological, Swampscott, Mass.) was coated at 2  $\mu$ g/mL in assay diluent (0.1% Bovine Gelatin, PBS, 0.05% Tween20) for two hours. C1q was detected with HRP conjugated sheep polyclonal anti-human C1q antibody (AbDSerotec) and quantified by measuring OD<sub>450</sub>.

The results are shown in FIG. 10. As shown in FIG. 10, the single and double Fc mutein antibodies produced by the materials and methods described herein had decreased C1q binding relative to those produced in mammalian cell culture or non-sialylated *Pichia pastoris* strains. C1q binding for the

antibodies produced from the single Fc muteins ( $\blacktriangle$  or  $\blacktriangledown$ ) were decreased 5-10 fold relative to the Her2 antibody while C1q binding for the double Fc mutein ( $\blacklozenge$ ) was virtually eliminated.

Both the *Pichia pastoris* Her2 antibody produced in a GFI5.0 strain (data not shown) and the Her2 antibody showed similar affinity to C1q.

#### EXAMPLE 13

##### Antibody Dependent Cellular Toxicity for Anti-Her2 and its Fc Muteins

Antibody dependent cellular toxicity (ADCC) was measured using a europium incorporation assay. The human ovarian adenocarcinoma line SKOV3 was cultured in McCoy's 5A media supplemented with 10% fetal bovine sera (FBS). Peripheral blood mononuclear cells (PBMC) were obtained from leucopaks. The PBMCs were subjected to Ficoll-Hypaque density centrifugation, washed in PBS supplemented with 2% FBS, and resuspended in McCoy's 5A media with 10% FBS. The Fc $\gamma$ RIIIa F158V genotype was determined for each individual donor. SKOV3 cells were labeled with EuDTPA. The cells were added to 96 well tissue culture plates at 5000/well and effector cells (PBMC) were added at 300,000/well (E:T 60:1). Antibodies were added at different concentrations and diluted across the plate. Controls included background, target and effector baseline counts, and 100% lysis wells. The PBMC mixtures with and without antibody were incubated for four hours at 37° C. Release of EuDTPA from lysed PBMC was determined by mixing 20  $\mu$ l of the supernatant with DELPHIA Enhancement Solution (Perkin Elmer, cat # 1244-105) to form a highly fluorescent chelate, which is measured using time resolved fluorometry. Triplicate wells were set up for each antibody dilution and the percentage of lysis was calculated according to the formula: ((experimental release-background)/(maximal release-background))-((natural cytotoxicity-background)/(maximal release-background)). The terms are defined as follows: experimental release represents the mean count for the target cells in the presence of effector cells and antibody, background represents the mean for the supernatant from the final wash after labeling the target cells, maximal release represents the mean for target cells incubated with DELPHIA lysis buffer (Perkin Elmer, cat # 4005-0010) and natural cytotoxicity represents the mean count for the target cells in the presence of effector cells. Data points were fit to a four parameter logistic regression model using Prism 5.0 software. All curves were constrained to share the same maximum, minimum, and slope.

Those of ordinary skill in the art would recognize and appreciate that the assays in Examples 10-12 can be readily adapted for requirements pertaining to any immunoglobulin molecule. Furthermore, an in vivo ADCC assay in an animal model can be adapted for any specific IgG using the methods of Borchmann et al., *Blood*, 102: 3737-3742 (2003); Niwa et al., *Cancer Research*, 64: 2127-2133 (2004).

The results are shown in FIG. 11. The single Fc muteins showed an ADCC activity profile similar to that for the Her2 antibody and approximately five fold lower than the *Pichia pastoris* Her2 antibody produced in a GFI 5.0 host. As illustrated in FIG. 11, ADCC activity is virtually absent for the antibody produced from the double Fc mutein, as expected from the Fc $\gamma$ RIIIa binding data.

## EXAMPLE 14

## Subcutaneous PK Study for Anti-Her2 and its Fc Muteins

The subcutaneous PK study was conducted in C57B6 mice. Antibody samples were administered subcutaneously at the dose of 1 mg/kg (n=3). Ten  $\mu$ l of blood was collected with a capillary tube at the collection time points of 1, 6, 24, 53, 77, 120, 192, 240, 288 and 360 hours and transferred to a microfuge tube containing 90  $\mu$ l of calcium and magnesium free PBS and 1.8 mg/ml K<sub>2</sub>EDTA. Human IgG levels were determined on a Gyros® Bioaffy (Uppsala, Sweden) workstation using a sandwich immunoassay, 100  $\mu$ g/ml biotinylated mouse monoclonal, anti-human kappa chain (BD Pharmingen, San Diego, Calif.) was used as the capture antibody and 12.5 nM ALEXA-647 labeled mouse monoclonal, anti-human Fc, Pan IgG (Southern Biotech, Birmingham, Ala.) was used as the detection antibody. The entire immunoassay was automated from capture antibody immobilization and analyte addition, to detection antibody addition and wash steps. Standards and QC were prepared in 5% mouse control plasma (EDTA) as 20 $\times$  stocks and diluted 1:20 into 5% mouse plasma in assay buffer (PBS, 0.01% Tween) prior to analysis. The linear range for accurate IgG concentration determination was established with spiked QC and found to be 5-5000 ng/ml. Accuracy and precision acceptability limits were  $\pm$ 20% with a lower limit of quantitation (LLOQ) of  $\pm$ 25%. Standards and QC could be frozen and thawed three times without significant loss of signal. Standards, QC and study samples were stored at -70° C. Study samples were thawed and diluted 1:20 into 5% mouse plasma in assay buffer and further diluted 1:10 if levels were outside the linear assay range. All standards, QC and study samples were assayed in duplicate and the average results reported. Concentrations were determined using a 5<sup>th</sup> parameter logistic curve fit. Pharmacokinetic parameters were calculated for each animal with WinNonlin using noncompartmental analysis of serum mAb concentration-time data (WinNonlin Enterprise Version 5.01, Pharsight Corp, Mountain View, Calif.).

As shown in FIG. 12, mice treated with *Pichia pastoris* Her2 with GFI5.0 glycosylation and Her2 have similar t<sub>1/2</sub> and serum concentrations, while mice treated with the Fc double mutein Her2, produced with GFI6.0 glycosylation, exhibited a higher C<sub>max</sub> (Maximum Concentration) and about a 30% increase in serum concentration as compared to mice treated with Her2 produced in CHO cells. Thus, of the three samples injected subcutaneously, the Fc double mutein antibody exhibited better bioavailability (absorption or exposure) based on the higher C<sub>max</sub> and serum concentrations.

## EXAMPLE 15

Another set of Fc $\gamma$  receptor binding assays for the anti-Her2 antibody of the invention and its Fc muteins were carried out as described in Example 11, and the results are shown in FIG. 13; and Tables 4 and 5.

TABLE 4

Comparison of Fc $\gamma$ R Binding Affinity to Her 2 Antibody					
Sample	Fc $\gamma$ RI	Fc $\gamma$ RIIa	Fc $\gamma$ RIIb/c	Fc $\gamma$ IIIa LF	Fc $\gamma$ IIIa LV
Her2 Ab	1.0	1.0	1.0	1.0	1.0
Pichia	0.6	0.4	0.9	11.3	2.8
Her 2 Ab					
F243A/V264A	0.2	0.02	No binding	0.04	0.4

TABLE 4-continued

Comparison of Fc $\gamma$ R Binding Affinity to Her 2 Antibody					
Sample	Fc $\gamma$ RI	Fc $\gamma$ RIIa	Fc $\gamma$ RIIb/c	Fc $\gamma$ IIIa LF	Fc $\gamma$ IIIa LV
F243A	0.6	0.1	0.3	8.4	3.3
V264A	0.6	0.03	0.2	3.6	1.4

Ratio calculation: STD EC50/anti-antigen mAb

Ratio &gt;1.0 higher affinity than Her2 Ab

Ratio &lt;1.0 lower affinity than Her 2 Ab

TABLE 5

Comparison of Fc $\gamma$ R Binding Affinity to Pichia Her 2 Antibody					
Sample	Fc $\gamma$ RI	Fc $\gamma$ RIIa	Fc $\gamma$ RIIb/c	Fc $\gamma$ IIIa LF	Fc $\gamma$ IIIa LV
Pichia	1.0	1.0	1.0	1.0	1.0
Her 2 Ab					
F243A/V264A	0.5	0.04	No binding	0.003	0.1
F243A	1.0	0.2	0.3	0.74	1.2
V264A	0.9	0.1	0.2	0.3	0.5

Ratio calculation: STD EC50/anti-antigen mAb

Ratio &gt;1.0 higher affinity than Pichia Her2 Ab

Ratio &lt;1.0 lower affinity than Pichia Her 2 Ab

## EXAMPLE 16

## ADCC Evaluation of Anti-HER2 Antibodies and Fc Muteins

ADCC analysis was performed using SKOV3 target cells (ATCC, Cat # HTB-77). On the day before the assay, primary NK effector cells (Biological Specialty, Cat # 215-11-10) were pelleted at 1000 rpm for 15 minutes and resuspended to 1 $\times$ 10<sup>6</sup> cells/ml in RPMI minus phenol red media (Invitrogen, catalog # 11835-030) supplemented with 10% FBS (Cellgro, Cat # 35-016-CV). The resuspended NK cells were incubated overnight at 37° C. under 5% CO<sub>2</sub>.

On the day of the assay, a flask of adhered SKOV3 target cells was washed with PBS, and the cells were detached using 3 ml trypsin (Cellgro, Cat # 25-053-CI) and incubation for 2-5 minutes at 37° C. The cells were collected with 23 ml RPMI minus phenol red media, 10% FBS and pipetted up and down to break apart clumps. The harvested cells were centrifuged at 1800 rpm for 5 minutes and resuspended to a concentration of 1 $\times$ 10<sup>7</sup> cells/ml with RPMI minus phenol red media, 10% FBS. The target cells (1 $\times$ 10<sup>7</sup> cells) were labeled with 100  $\mu$ Ci of Chromium-51 (5 mCi Sodium chromate in normal saline, Perkin Elmer, Cat # NEZ03005MC). Target cells were incubated at 37° C. for one hour with shaking every 15 minutes. Cells were centrifuged at 1800 rpm for 2 minutes and resuspended in one ml RPMI minus phenol red media, 10% FBS. Cells were washed two additional times with one ml RPMI minus phenol red media, 10% FBS with centrifugation at 1800 rpm for 2 minutes between each wash. After the final wash, the labeled target cells were resuspended in RPMI minus phenol red media, 10% FBS to a final concentration of 2.5 $\times$ 10<sup>5</sup> cells/ml.

Test antibodies used in these assays were anti-HER2 mAb produced in GFI 5.0, anti-HER2 mAb F243A produced in GFI 6.0 (GS6.0/F243A), anti-HER2 mAb V264A produced in GFI 6.0 (GS6.0/V264A), and anti-HER2 mAb F243A/V264A produced in GFI 6.0 (GS6.0/F243A/V264A). While the SKOV3 target cells were being labeled, test antibodies were diluted using a 3-fold serial titration (starting at 1  $\mu$ g/ml) in RPMI minus phenol red media, 10% FBS in a polystyrene 96-well plate (Costar, Cat # 353077). To the wells of a separate 96-well assay plate, 100  $\mu$ l of Cr-51 labeled SKOV3

target cells (=25,000 cells) were transferred. After the antibody dilution plate was prepared, 10  $\mu$ l of each dilution was transferred to the 96-well assay plate containing the labeled target cells. For the controls, 10  $\mu$ l of Triton-X100 (10% stock, Fluka Analytical, Cat # 93443) or 10  $\mu$ l of media was added to "Max lysis" or "spontaneous release" control wells, respectively. Each antibody dilution was tested in duplicate, while 6 replicates of each control were tested.

Primary NK cells were pelleted at 1200 rpm for 5 minutes and gently resuspended to  $2.5 \times 10^6$  cells/ml in RPMI minus phenol red media, 10% FBS. To all sample wells and "no antibody" control wells (i.e., excluding "spontaneous release" and "max lysis" controls), 100  $\mu$ l of NK cells (=250,000 cells) was added for an effector:target ratio of 10:1. The assay plate was incubated at 37° C. under 5% CO<sub>2</sub> for four hours. After the incubation, the assay plate was centrifuged at 300 rpm for 5 minutes. Thirty  $\mu$ l of the supernatants was added to 250  $\mu$ l of Microscint 20 (Perkin Elmer, Cat # 6013621) in a 96-well Picoplate (Perkin Elmer, Cat # 6005185). The Cr-51 release was measured in a Packard Top Count scintillation counter.

Percent lysis was calculated as: ((ADCC experimental release - Spontaneous release) / (Max release - Spontaneous release)) \* 100

The results of these assays are presented in FIG. 14. As shown in FIG. 14, the F243A single mutein antibody and the V264A single mutein antibody had a 5-10 fold reduction in ADCC activity when compared to the parent (wildtype) antibody; while the F243A/V264A double mutein antibody had more than 100 fold reduction in ADCC activity when compared to the parent (wildtype) antibody.

#### EXAMPLE 17

Statistical Analysis of OF ADCC Data of Anti-HER2 Antibody and its Fc Muteins

The objective of the study was to determine if the percentage lysis for the double mutant variant is synergistic with respect to the two single mutant variants. The determination is made by comparing the ED50 (antibody level corresponding to 50% lysis) between the double mutant variant (group 4) and the assumed additive reference curve (shown in FIG. 15 and Table 6). Since the lower limit of the ED50 for the double mutant curve is above the top antibody level assayed and is well above the upper limit of the ED50 for additive reference curve we conclude that the effect of the double mutation is much more than additive.

Statistical Methods and Results:

The objective was to determine whether the double mutation variant of the Herceptin antibody (GS6.0/F243A/V264A) demonstrates a synergistic effect compared to the two single mutation variants (GS6.0/F243A and GS6.0/V264A). There are four antibody groups: Group 1 (GS5.0), Group 2 (GS6.0/F243A), Group 3 (GS6.0/V264A) and Group 4 (GS6.0/F243/V264A).

Data from three donors is run on separate 96-well plates. The observations from each plate are normalized by converting the values to percentage of lysis as follows:

$$\% \text{ Lysis} = 100 \times \left( \frac{O_{rc} - O_N}{O_P - O_N} \right)$$

where:  $O_{rc}$  is the observed response in the  $r^{th}$  row and  $c^{th}$  column of each plate.

$O_N$  is the average response of the negative controls for each plate.

$O_p$  is the average response of the positive controls for each plate.

Duplicate % lysis values for each antibody-level and group are then averaged.

The data from all four groups and all three donors are modeled jointly. We assume the 'antibody level-response' relationship follows a sigmoid Hill Equation (Eq. 1). We use (100% minus % lysis) as our response variable (Y) and the log antibody-levels as the explanatory variable (X). In order to make comparisons about the potency of the various mutations and fit the models, some assumptions about the model parameters for each group are made.

The form of the model used assumes:

1. The span (a) and the plateau (d), for all the 4 treatment groups are the same.
2. The same slope for all the treatment groups except the GS5.0 group which is allowed to have a different slope.
3. The EC50 parameters ( $\gamma$ ) are different for the different groups.

The Hill Equation:

$$Y_{ij} = \frac{a}{1 + \left( \frac{X_{ij}}{\gamma + \gamma_j} \right)^{\beta_0 + \beta_j}} + d \quad (\text{Eq. 1})$$

With the constraints:  $\gamma_1=0$ ,  $\beta_1=0$  and  $\beta_2=\beta_3=\beta_4=\beta$

Here:

$Y_{ij}$  is the  $i^{th}$  response (100-% Lysis) for the  $j^{th}$  treatment  
 $X_{ij}$  is the antibody level (on the log scale) corresponding to  $Y_{ij}$   
 $j=1$  for GS5.0  $j=2$  for GS6.0/F243A  
 $j=3$  for GS6.0/V264A  $j=4$  for GS6.0/F243A/V264A

It is further assumed that if the two single mutants (GS6.0/F243A and GS6.0/V264A) were additive then the equation for the combined effects would be:

$$Y_{i(\text{additive})} = \frac{a}{1 + \left( \frac{X_{i(\text{additive})}}{(\gamma + \gamma_2 + \gamma_3)} \right)^{\beta_0 + \beta}} + d \quad (\text{Eq. 2})$$

with the parameters a, d,  $\gamma$ ,  $\gamma_2$ ,  $\gamma_3$ ,  $\beta$  and  $\beta_0$  being the same as in Eq. 1.

The models (Eq. 1 and Eq. 2) are fit using PROC NLIN in SAS v9.2. The observed data values and the fitted curves for the four groups, as well as the assumed additive reference curve for the two single mutation groups combined are displayed in the FIG. 15. The ED50's along with their confidence intervals (on the original scale) are given in Table 6.

TABLE 6

Groups	ED50	95%	
		Confidence Interval	
Group 1: GS5.0 (anti-Her 2 mAb produced in GFI 5.0)	0.011	0.0085	0.0131
Group 2: GS6.0/F243A (anti-Her 2 mAb F243A produced in GFI 6.0)	0.059	0.0457	0.0769
Group 3: GS6.0/V264A (anti-Her 2 mAb V264A produced in GFI 6.0)	0.072	0.0558	0.0942
Group 4: GS6.0/F243A/V264A (anti-Her 2 mAb F243A/V264A double mutein produced in GFI 6.0)	>1 *	>1 *	>1 *
Group 2&3 combined: Additive Reference Curve	0.407	0.3031	0.5455

\* Estimated value is greater than the highest antibody level in the experiment.

In order to assess the effects of GS6.0/F243A/V264A compared to the two single mutations GS6.0/F243A and GS6.0/V264A; the ED50 of the group 4 is compared with the ED50 of the additive reference curve. If the confidence intervals are non-overlapping then one can conclude that the two quantities are statistically different.

The 95% confidence interval for the ED50 of the additive reference curve is (0.3031, 0.5455) while the estimate and confidence intervals for the ED50 of group 4 are all higher than 1 (the estimated values are outside the observed range of antibody-levels). The lower 95% confidence limit for the ED50 of the double mutation is >1, which is much higher than upper 95% confidence limit for the ED50 of the reference additive curve (group 2&3 combined). Hence one can conclude the two quantities are significantly different.

The above comparison of confidence intervals of ED50's is equivalent to comparing  $\gamma_4$  versus the sum ( $\gamma_2 + \gamma_3$ ), i.e. testing the hypothesis that  $H_0: \gamma_4 = (\gamma_2 + \gamma_3)$  versus the alternative that  $H_a: \gamma_4 > (\gamma_2 + \gamma_3)$

Let  $SSE_{reduced}$  and  $SSE_{full}$  is the residual sum of squares from the reduced model and the full model respectively. The full model is obtained by fitting Eq. 1 to the data while the reduced model is obtained by substituting  $\gamma_4$  in Eq. 1 with ( $\gamma_2 + \gamma_3$ ).

The hypothesis:

$$H_0: \gamma_4 = (\gamma_2 + \gamma_3) \text{ versus } H_a: \gamma_4 > (\gamma_2 + \gamma_3)$$

The Test Statistic:

$$F_{obs} = \left\{ \frac{SSE_{reduced} - SSE_{full}}{DF_{reduced} - DF_{full}} \right\} / MSE_{full}$$

The p-value:

$$P(F_{obs} > F_{(DF_{reduced}-DF_{full}), DF_{full}}) < 0.0001$$

Since the p-value is <0.0001 we can reject  $H_0$  and conclude that  $\gamma_4 > (\gamma_2 + \gamma_3)$ .

The data and model based comparisons indicate that the double mutation GS6.0/F243A/V264A has a significantly lower % lysis, than the hypothesized combined effects of the two single mutations. The effect of the double mutation is much more than an additive effect of the single mutations.

References Relating to this Example:

- [1] SAS (r) Proprietary Software 9.2 (TS2M2), SAS Institute Inc., Cary, N.C., USA.
- [2] 'Application of the Four-Parameter Logistic Model to Bioassay: Comparison with Slope Ratio and Parallel Line Models'; Aage Vølund, *Biometrics*, Vol. 34, No. 3 (September 1978), pp. 357-365

#### EXAMPLE 18

Construction of Anti-TNF $\alpha$  Fc Muteins and ADCC Evaluation

The preparation of a double Fc muteins (F243A/V264A) of an anti-TNF monoclonal antibody in *Pichia pastoris* was carried out using the sequences and protocols listed below. The heavy and light chain sequences of the parent (wildtype) anti-TNF $\alpha$  antibody are set for the in SEQ ID NOs:10 and 11. The sequence of the heavy chain of the double mutein anti-TNF $\alpha$  antibody is set forth in SEQ ID NO:12. The light chain sequence of the wt and double mutein anti-TNF $\alpha$  antibodies are identical.

The signal sequence of an alpha-mating factor predomain (SEQ ID NO:8) was fused in frame to the end of the light or

heavy chain by PCR fusion. The sequence was codon optimized and synthesized by Genscript (GenScript USA Inc., 860 Centennial Ave. Piscataway, N.J. 08854, USA). Both heavy chain and light chain were cloned into antibody expression vector as similar way of constructing anti-HER2 IgG1 and its Fc muteins.

The heavy and light chains with the fused signal sequence of IgG1 and its muteins were cloned under *Pichia pastoris* AOX1 promoter and in front of *S. cerevisiae* Cyc terminator, respectively. The expression cassette of the completed heavy and light chains was put together into the final expression vector. Genomic insertion into *Pichia pastoris* was achieved by linearization of the vector with Spe1 and targeted integration into the Trp2 site. Plasmid pGLY6964 encodes wildtype anti-TNF $\alpha$  IgG1 antibody. Plasmid pGLY7715 encodes the anti-TNF alpha IgG1F243A/V264A double mutein.

Glycoengineered *Pichia* GFI5.0 YGLY8316 and GFI6.0 YGLY 22834 Hosts for Producing Anti-TNF $\alpha$  its Fc Muteins

The genotype of the GFI5.0 strain YGLY16786 used for the expression of the anti-TNF $\alpha$  antibodies and its Fc muteins, is as follows:ura5 $\Delta$ ::ScSUC2 och1 $\Delta$ ::lacZ bmt1 $\Delta$ ::lacZ bmt2 $\Delta$ ::lacZbmt3 $\Delta$ ::lacZbmt4 $\Delta$ ::lacZ/KIMNN2-2 mnn4L1 $\Delta$ ::lacZ/MmSLC35A3 pno1 $\Delta$ ::lacZ ADE1::lacZ/FB8/NA10/MmSLC35A3 his1::lacZ-URA5-lacZ/XB33/Sp-25 GALE/DmUGT arg1::HIS1/KD53/TC54 PRO1::ARG1/AOX1-ScMFpreTrMNS1PRO1::ARG1/AOX1-ScMFpreTrMNS1.

The genotype of the engineered *Pichia pastoris* GFI 6.0 strain YGLY23423 used for the expression of the anti-TNF $\alpha$  Fc DM mutein is as follows: ura5 $\Delta$ ::ScSUC2 och1 $\Delta$ ::lacZ bmt2 $\Delta$ ::lacZ/KIMNN2-2 mnn4L1 $\Delta$ ::lacZ/MmSLC35A3 pno1  $\Delta$  mnn4 $\Delta$ ::lacZ ADE1::lacZ/NA10/MmSLC35A3/FB8his1 $\Delta$ ::lacZ/ScGAL10/XB33/DmUGT arg1 $\Delta$ ::HIS1/KD53/TC54bmt4 $\Delta$ ::lacZ bmt1 $\Delta$ ::lacZ bmt3 $\Delta$ ::lacZ TRP2: 35 ARG1/MmCST/HsGNE/HsCSS/HsSPS/MmST6-33ste13 $\Delta$ ::lacZ/TrMDS1 dap2 $\Delta$ ::Nat<sup>R</sup> TRP5:Hyg<sup>R</sup>MmCST/HsGNE/HsCSS/HsSPS/MmST6-33 Vps10-1 $\Delta$ ::AOX1p\_LmSTT3d.

The cells were transformed, screened and purified as indicated in Examples 4-7.

Antibody Dependent Cell Cytotoxicity (ADCC) Evaluation of Anti-TNF $\alpha$  Fc Muteins

ADCC analysis was performed using Jurkat FlpIn target cells stably-expressing a non-cleavable variant of TNF $\alpha$  which has the first 12 amino acids removed—Jurkat FlpIn TNF $\alpha$  ( $\Delta$ 1-12) target cells. These cells were prepared by transfecting Jurkat FlpIn human T-cell leukemia cells (Invitrogen) with a nonsecretable cell surface mutant of TNF $\alpha$  ( $\Delta$ 1-12 TNF $\alpha$   $\tilde{q}$ ). Perez et al., *Cell* 63; 251-258 (1990). The  $\Delta$ 1-12 TNF $\alpha$  DNA was cloned into pcDNA5 vector (Invitrogen) to use for the transfection. Expression of cell surface TNF $\alpha$  was confirmed by flow cytometry.

On the day before the assay, primary NK effector cells (Biological Specialty, Cat # 215-11-10) were pelleted at 1200 rpm for 12 minutes and gently resuspended to approximately  $1-1.5 \times 10^6$  cells/ml in RPMI minus phenol red media (Invitrogen, catalog # 11835-030) supplemented with 10% heat-inactivated FBS (Sigma, Cat # F4135), 10 mM Hepes (Gibco, Cat # 15630), 2 mM L-glutamine (Cellgro, Cat # 25-005-CI), and  $1 \times$  penicillin/streptomycin (Cellgro, Cat # 30-002-CI). The resuspended NK cells were incubated overnight at 37 $^\circ$  C. under 5% CO<sub>2</sub>.

On the day of the assay, Jurkat FlpIn TNF $\alpha$  ( $\Delta$ 1-12) target cells were centrifuged at 1200 rpm for 5 minutes, and the cell pellet was gently resuspended to a concentration of  $2 \times 10^6$  cells/ml in RPMI minus phenol red media supplemented with 5% heat-inactivated FBS. The cells were labeled with 25  $\mu$ l

DELFLIA BATDA labeling reagent (from DELFLIA EuTDA Cytotoxicity kit, Perkin Elmer, Cat # AD0116). Cells were mixed gently and incubated at 37° C. for 20 minutes with gentle mixing every 10 minutes. Cell volume was adjusted to 30 ml with DPBS containing 1.5 mM probenidol (Invitrogen, Cat # P36400) and centrifuged at 1200 rpm for 5 minutes. Cells were washed three times with 30 ml DPBS containing 1.5 mM probenidol with centrifugation at 1200 rpm for 5 minutes between each wash. After the final wash, the labeled target cells were resuspended in RPMI minus phenol red media supplemented with 5% heat-inactivated FBS and 1.5 mM probenidol to a final concentration of  $2.5 \times 10^5$  cells/ml.

Test antibodies used in the assay were: anti-TNF IgG1 wildtype antibody (designated "GFI774") produced in GFI 5.0, non-sialylated anti-TNF IgG1 antibody (GFI774) F243A/V264A produced in GFI 5.0, and sialylated anti-TNF IgG1 antibody (GFI774) F243A/V264A produced in GFI 6.0. The non-sialylated anti-TNF IgG1 antibodies—F243A/V264A—produced in GFI 5.0 comprise the following glycoforms: 84% G2, 2% G1 and 14% hybrid N-glycans. The sialylated anti-TNF IgG1 antibodies—F243A/V264A—produced in GFI 6.0 comprise the following glycoforms: 27% A1, 50.6% A2, and 5.7% A1 hybrid (overall, more than 83% of the N-glycans are sialylated).

While the Jurkat FlpIn TNF $\alpha$  ( $\Delta$ 1-12) target cells were being labeled, 2 $\times$  concentrations of antibodies were diluted using a 3-fold serial titration (starting at 40  $\mu$ g/ml) in RPMI minus phenol red media supplemented with 5% heat-inactivated FBS and 1.5 mM probenidol in a 96-well polypropylene, round-bottom plate (Costar, Cat # 5699; lids—Costar Cat # 3931). After the dilution plate was prepared, 100  $\mu$ l of each 2 $\times$  dilution was transferred to a new 96-well round-bottom polypropylene plate (100  $\mu$ l of media only was transferred for the "no antibody" controls). RPMI minus phenol red media supplemented with 5% heat-inactivated FBS and 1.5 mM probenidol was also transferred to the 96-well plate for the "spontaneous release" and "max lysis" controls (150  $\mu$ l per well) and the "background" controls (200  $\mu$ l per well). Each antibody dilution was tested in duplicate, while each control was tested in quadruplicate.

To all wells except "background" controls, 50  $\mu$ l of Europium-labeled Jurkat FlpIn TNF $\alpha$  ( $\Delta$ 1-12) cells (=12,500 cells) were added and mixed gently. Primary NK cells were pelleted at 1200 rpm for 12 minutes and gently resuspended to  $2.5 \times 10^6$  cells/ml in RPMI minus phenol red media supplemented with 5% heat-inactivated FBS and 1.5 mM probenidol. To all sample wells (i.e., excluding "spontaneous release", "max lysis", and "background" controls), 50  $\mu$ l of NK cells (=125,000 cells) was added, for an effector:target ratio of 10:1. Samples were mixed gently, and the assay plate was incubated at 37° C. for two hours. After 1 hour and 15 minutes, 10  $\mu$ l of 20% Triton-X100 (Pierce Surfact-Amps X-100, Cat # 28314) or 10  $\mu$ l of media was added to "max lysis" or "spontaneous release" control wells, respectively. The plate was incubated at 37° C. for an additional 45 minutes (total incubation time was 2 hours). While plates were incubating, DELFLIA Europium Solution (from DELFLIA EuTDA Cytotoxicity kit) was equilibrated at room temperature. After the two hour incubation, the assay plate was centrifuged at 1500 rpm for 5 minutes. Twenty  $\mu$ l of the supernatants was transferred to a white, flat-bottom clear plate (from DELFLIA EuTDA Cytotoxicity kit or Costar, Cat # 3632) taking care that no bubbles were introduced. To each well, 200  $\mu$ l of DELFLIA Europium Solution was added, and the plate was covered with an aluminum foil seal. The assay plate was incubated at room temperature for 15 minutes with gentle

shaking. Fluorescence was measured using a Perkin Elmer Envision instrument set up for reading Europium.

Percent lysis was calculated as follows:

- 1) the average of the background control was subtracted from all raw values,
- 2) the % of ADCC activity was calculated as: ((ADCC experimental value - Spontaneous release) / (Max Lysis - Spontaneous release)) \* 100 and
- 3) the final % lysis values were reported as % ADCC activity from step 2 minus "No Antibody" control.

The results of these assays are presented in FIG. 16. As shown in FIG. 16, the non-sialylated and sialylated anti-TNF $\alpha$  IgG1-F243/V264A double mutein had more than 1000 fold reduction in ADCC as compared to the parent (wildtype) polypeptide produced in GS5.0.

#### EXAMPLE 19

##### Complement Dependent Cytotoxicity (CDC) Evaluation of Anti-TNF $\alpha$ Double Fc Muteins

CDC analysis was performed using HEK293 FlpIn cells stably-expressing a non-cleavable variant of TNF  $\alpha$  which has the first 12 amino acids removed. HEK293 FlpIn TNF $\alpha$  ( $\Delta$ 1-12) cells were grown in tissue culture flasks in Dulbecco's Minimal Essential Media (DMEM) minus phenol red (Gibco, Cat # 21063) supplemented with 10% heat-inactivated FBS (Sigma, Cat # F4135), 100  $\mu$ g/ml Hygromycin B (Cellgro, Cat # 30-240-CR), and L-glutamine (Cellgro, Cat # 25-005-CI) to 70% confluence. Cells were treated with 2 ml trypsin (Cellgro, Cat # MT25-053-CI), harvested with 8 ml DMEM minus phenol red media, 10% heat-inactivated FBS, and centrifuged at 1200 rpm for 10 minutes. The supernatant was removed and cell pellet was resuspended in DMEM minus phenol red media, heat-inactivated 10% FBS to a concentration of  $4 \times 10^5$  cells/ml. The cells were plated in 100  $\mu$ l (40,000 cells) per well in a 96-well black with clear bottom plate (Costar, Cat # 3603) and incubated overnight at 37° C. under 5% CO<sub>2</sub>.

Test antibodies used in the assay were: anti-TNF IgG1 antibody (designated "GFI774") produced in GFI 5.0, anti-TNF IgG1 antibody (GFI774) F243A/V264A produced in GFI 5.0, and anti-TNF IgG1 antibody (GFI774) F243A/V264A produced in GFI 6.0. (These antibodies are described in Example 18.) On the day of the assay, the media was aspirated with a multichannel pipettor from the wells of the 96-well plate and replaced with 50  $\mu$ l of DMEM minus phenol red media, 1 $\times$  penicillin/streptomycin. A 2-fold serial titration of test antibody (starting at 30  $\mu$ g/ml) was prepared in DMEM minus phenol red media containing 1 $\times$  penicillin/streptomycin, 10  $\mu$ g/ml anti-human CD55 mouse IgG1 monoclonal antibody (IBGRL Research Products, Clone BRIC216, Cat # 9404P) and 10  $\mu$ g/ml anti-human CD59 mouse IgG2b monoclonal antibody (IBGRL Research Products, Clone ERIC 229, Cat # 9409P). To the appropriate assay plate wells, 50  $\mu$ l of the diluted antibody was added. Assay negative controls were assay media alone and human IgG (Jackson ImmunoResearch, Cat # 009-000-003) diluted in the assay DMEM minus phenol red media (containing CD55 and CD59 antibodies). The assay lysis control was 0.25% Triton X100 (10% stock, Fluka, Cat # 93443) diluted in the assay DMEM minus phenol red media (containing CD55 and CD59 antibodies). Each test antibody concentration was tested in duplicate while the assay controls were tested in triplicate. The assay plate containing test samples was mixed by tapping lightly, and the plate was incubated for approximately 10 minutes at 37° C. while the human complement sera was being prepared. Three ml of human complement



(QUIDEL, Cat # A113) was diluted 1:2 with 3 ml of DMEM minus phenol red media containing 1× penicillin/streptomycin, and 50 µl of the diluted complement was added to the assay plate wells. The assay plate was mixed by tapping lightly, and the plate was incubated for 4 hours at 37° C. under 5% CO<sub>2</sub>. A solution of 40% alamar blue (Biosource, Cat # DAL1100) was prepared by diluting 100% stock with DMEM minus phenol red media containing 1× penicillin/streptomycin, and 50 µl of the diluted alamar blue solution was added to the assay plate wells (=10% final). The assay plate was mixed by tapping lightly, and the plate was incubated overnight (15-20 h) at 37° C. under 5% CO<sub>2</sub>. The next day, the assay plate was incubated for 10 minutes on a shaker at room temperature and fluorescence was read at excitation 544 nm, emission 590 nm. Percent CDC was calculated as:  $(1 - (\text{Sample raw fluorescence unit (RFU)} - \text{Triton RFU}) / (\text{Media RFU} - \text{Triton RFU})) * 100$

The results of these assays are presented in FIG. 17. As shown in FIG. 17, the non-sialylated and sialylated anti-TNF $\alpha$  IgG1-F243/V264A double mutein had about a 10 fold reduction in CDC activity as compared to the parent (wild-type) antibody.

#### EXAMPLE 20

Effect of the Anti-TNF $\alpha$  Antibody and its Fc Muteins in a Collagen-Antibody Induced Arthritis (AIA) Model

MODEL INDUCTION: AIA (Antibody induced arthritis) is induced with a commercial Arthrogen-CIA<sup>®</sup> arthritogenic monoclonal antibody (purchased from Chondrex) consisting of a cocktail of 5 monoclonal antibodies, clone A2-10 (IgG2a), F10-21 (IgG2a), D8-6 (IgG2a), D1-2G (IgG2b), and D2-112 (IgG2b), that recognize the conserved epitopes on various species of type II collagen.

ANIMALS: 10 week old B10.RIII male mice which are susceptible to arthritis induction without additional of co-stimulatory factors were used. These animals were purchased from Jackson Laboratory.

CLINICAL SCORING: Paw swelling was measured daily post-induction of arthritis. The severity of the disease was graded on a 0-3 scale per paw as follows: 0, normal; 1, swelling of one digit; 2, swelling of two or more digits; 3, swelling of the entire paw. The maximal clinical score per mouse is 12.

STUDY DESIGN: Arthritis was induced by passive transfer of 3 mg of anti-CII mAb pathogen cocktail IV on day 0. Groups of Mice were treated subcutaneously with following reagents:

Group/Reagent	Lot	Dose
A. Naïve	***	***
B. Isotype IgG1	78ABY	33 mpk
C. Asialyated Anti-TNF	36ADV	33 mpk
D. $\alpha$ 2,6 Sialyated Anti-TNF	37ADV	33 mpk
E. mTNFR-Ig	82ABW	33 mpk
F. GAMMAGARD	84ADU	33 mpk
G. GAMMAGARD	84ADU	1000 mpk
H. HUMIRA	85ADU	33 mpk

Group n=5 for all groups except Group A and H which have n=3.

An isotype IgG1 antibody was used as a control. The antibody bound to mouse anti-hexon and had the designation "27F11".

The sample identified as "Asialyated Anti-TNF" corresponds to the anti-TNF antibody as described in Example 18

produced in GFI 5.0 comprising the F243A/V264A mutations. The sample identified as " $\alpha$ 2,6 Sialyated Anti-TNF" corresponds to the anti-TNF antibody described in Example 18 produced in GFI 6.0 comprising the F243A/V264A mutations. The non-syalated anti-TNF IgG1 antibodies—F243A/V264A—produced in GFI 5.0 comprise the following glycoforms: 84% G2, 2% G1 and 14% hybrid N-glycans. The syalated anti-TNF IgG1 antibodies—F243A/V264A—produced in GFI 6.0 comprise the following glycoforms: 27% A1, 50.6% A2, and 5.7% A1 hybrid (overall, more than 83% of the N-glycans are syalated).

mTNFR-Ig is an immunoadhesin (anti-TNF receptor Ig-fusion protein) comprising the extracellular domain of mTNFR2 connected to mIgG1Fc starting at the hinge and spanning the CH2 and the CH3 regions, and comprising the amino acid sequence of SEQ ID NO:16 produced in CHO cells.

GAMMAGARD liquid was purchased from Baxter Corp. HUMIRA was purchased from Abbott Labs. HUMIRA comprise asyalated N-glycans with very little terminal galactose.

All groups of mice were dosed on day minus 1 and day 7 with the exception of mTNFR-Ig, which received a total of three doses at days minus 1, +3, and +7. The Clinical Score was monitored for 14 days.

The results of these experiments are shown in FIGS. 18A and 18B. In this study, GAMMAGARD did not demonstrate clinical efficacy. Mu-TNF-Ig showed good protection from disease in 4 out of 5 mice. HUMIRA had very similar disease kinetics as the control IgG1. Asialyated Anti-TNF showed some dampening of disease. The  $\alpha$  2,6 sialyated Anti-TNF showed good disease protection in 5 out of 5 mice, and scores were comparable to anti-TNF therapy.

GENE EXPRESSION ANALYSIS: Expression of inflammatory and bone remodeling genes were determined by RT/PCR analysis of hind paws from isotype,  $\alpha$ 2,6 sialyated mAb or mTNFR-Ig treated mice (n=4 per group). To perform the quantitative PCR, total RNA was isolated from hind paws using RNA STAT-60 (Tel-Test, Friendswood, Tex., USA). Total RNA (5 µg) was subjected to treatment with DNase (Roche). DNase-treated total RNA was reverse-transcribed using Superscript II (Gibco/BRL). Primers were designed using Primer Express (PE Biosystems), or obtained commercially from Applied Biosystems.

Real-time quantitative PCR on 10 ng of cDNA from each sample was performed using one of two methods. In the first method, two gene-specific unlabelled primers were utilized at 400 nM in a Perkin Elmer SYBR green real-time quantitative PCR assay utilizing an ABI 5700 Instrument. In the second method, two unlabelled primers at 900 nM each were used with 250 nM of FAM-labelled probe (Applied Biosystems) in a TAQMANTM real-time quantitative PCR reaction on an ABI 7700 sequence detection system. The absence of genomic DNA contamination was confirmed using primers that recognize genomic regions of the CD4 promoter-samples with detectable DNA contamination by real-time PCR were excluded from the study. Ubiquitin levels were measured in a separate reaction and used to normalize the data by the  $\Delta$ - $\Delta$  Ct method, using the mean cycle threshold (Ct) value for ubiquitin and the genes of interest for each sample; the equation  $1.8 e^{(Ct \text{ ubiquitin} - Ct \text{ gene of interest})} \times 10^4$  was used to obtain the normalized values. The results are shown in Table 7. Data shown is fold-increase of inflammatory/bone remodeling gene expression over that of gene expression in hind paws of naïve control mice.

TABLE 7

Fold induction over naïve mice			
Gene	Isotype 33 mpk	$\alpha$ 2,6 SA Humira 33 mpk	mTNFR-Ig 33 mpk
IL-1b	14.43	0.90	0.43
IL-6	18.45	0.75	0.77
Tnfsf2 - Tnfa	2.63	0.98	0.70
Tnfsf11 - RankI	10.94	1.27	0.67
Tnfsf11a - Rank	2.21	0.86	0.74
MDL-1 long	5.25	1.82	0.88
F4/80	3.20	1.75	0.56
Cd11b	3.57	1.35	1.07
Cd14	2.48	1.53	0.52
Dap12	4.36	1.33	0.90
TIMP-1	15.85	1.44	1.38
PU.1 - Sfp1	3.88	1.09	0.79
Trap - Acp5	10.19	1.85	1.17
Ccl2 - Mcp1	8.01	1.24	0.87
Ccl3 - Mip1a	4.06	1.09	0.58
Cxcl1 - Groa	16.39	1.65	0.69
Cxcl2 - Grob	5.33	0.50	0.32
Atp6v0d2	17.45	2.05	1.74
Mmp9	13.53	1.88	1.32
Fcgr1 - Cd64	4.51	1.04	0.68
Fcgr2b - Cd32a	2.75	1.11	0.71
Fcgr3	0.57	0.74	0.66
Fcgr4	6.54	1.38	1.04
Ctsk	6.85	1.65	0.91
Calcr	1.80	0.49	0.94
Cd68 - Scard1	3.19	1.29	0.94
Itgb2 - Cd18	3.90	1.33	0.88

## EXAMPLE 21

Effect of the Anti-TNF $\alpha$  Antibody or its Fc Muteins in a Collagen-Antibody Induced Arthritis (AIA) Model

The experiment described in Example 20 was repeated as described therein, with the exception that GAMMAGARD was administered intravenously (while all other reagents were administered subcutaneously).

STUDY DESIGN: Arthritis was induced as described in Example 20.

All groups of mice were dosed on day minus 1 with the exception of mTNFR-Ig, which received a total of two doses at days minus 1 and +3. The Clinical Score was monitored for 7 days.

Groups of Mice were treated with following reagents:

Group/ Reagent	Lot	Dose
Naïve	***	***
Isotype IgG1	78ABY	33 mpk
Asialyated Anti-TNF	36ADV	33 mpk
$\alpha$ 2,6 Sialyated Anti-TNF	37ADV	33 mpk or 6.6 mpk
$\alpha$ 2,3 Sialyated Anti-TNF	19ADX	33 mpk
mTNFR-Ig	82ABW	33 mpk
GAMMAGARD	84ADU	1000 mpk
HUMIRA	85ADU	33 mpk
18 ADX PNG Humira	18ADX	33 mpk
20 ADX No Glyco	20ADX	33 mpk

Group n=5 for all groups

The reagents: "Isotype IgG1", "Asialyated Anti-TNF", " $\alpha$ -2,6 Sialyated Anti-TNF, mTNFR-Ig, GAMMAGARD and HUMIRA were described in Example 20.

The reagent identified as " $\alpha$ -2,3 Sialyated Anti-TNF" corresponds to the anti-TNF antibody described in Example 18 produced in GFI 6.0 comprising the F243A/V264A, which was in vitro treated with neuraminidase to eliminate the  $\alpha$ 2,6 linked sialic acid, and further in vitro treated with  $\alpha$ 2,3 sia-

lyltransferase. Briefly, the purified antibody (4-5 mg/ml) was in the formulation buffer comprising 6.16 mg sodium chloride, 0.96 mg monobasic sodium phosphate dehydrate, 1.53 mg dibasic sodium phosphate dihydrate, 0.30 mg sodium citrate, 1.30 mg citric acid monohydrate, 12 mg mannitol, 1.0 mg polysorbate 80 per 1 ml adjusted to pH to 5.2. Neuraminidase (10 mU/ml) was added to antibody mixture and incubated at 37 C for at least 5 hrs or until desialylation reached completion. The desialylated material was applied onto CaptoMMC (GE Healthcare) column purification to remove neuraminidase and reformulated in Sialyltransferase buffer (50 mM Hepes pH 7.2 150 mM NaCl, 2.5 mM CaCl<sub>2</sub>, 2.5 mM MgCl<sub>2</sub>, 2.5 mM MnCl<sub>2</sub>) at 4 mg/ml. Mouse  $\alpha$ 2,3 sialyltransferase recombinant enzyme expressed in *Pichia* and purified via his-tag was used for  $\alpha$  2,3 sialylic acid extension. The enzyme mixture was formulated in PBS in the presence of Protease Inhibitor Cocktail (Roche™, cat # 11873580001) at 1.2 mg/ml. Prior to the sialylation reaction, pepstatin (50 ug/ml), chymostatin (2 mg/ml) and 10 mM CMP-Sialic acid were added to the enzyme mixture followed by sterilization through 0.2  $\mu$ m filter. One ml of enzyme mixture was added to 10 ml desialylated material. The reaction was carried out at 37 C for 8 hrs. The sialylation yield was confirmed by mass determination by ESI-Q-TOF. The final material was purified using MabSelect (GE Healthcare) and formulated in the buffer described above and sterile-filtered (0.2  $\mu$ m membrane).

The reagent identified as "18 ADX PNG Humira" corresponds to the anti-TNF antibody described in Example 18 produced in GFI 6.0 comprising the F243A/V264A which was in vitro treated with PNGase F to remove all N-glycans. The PNGase F enzyme was obtained Prozyme, Inc., and used with a stoichiometry of 2  $\mu$ l of commercial enzyme to 4 mg of IgG1 at pH 7.4. The digested material was purified using Mabselect (GE Healthcare). The final material was formulated and sterile-filtered (0.2  $\mu$ m membrane).

The reagent identified as "20 ADX No Glyco" corresponds to the wildtype anti-TNF antibody described in Example 18, comprising a single mutation at position 297 which results in no glycosylation, produced in a GFI5.0 YGLY8316. The strain producing this antibody was clarified by centrifugation for 15 min at 13,000 g in a Sorvall Evolution RC (kendo, Asheville, N.C.). The capture step was performed using MabSelect (GE Healthcare) followed by a polishing step using Capto MMC (GE Healthcare). The final material was formulated and sterile-filtered (0.2  $\mu$ m membrane).

The results of these experiments are shown in FIGS. 19A and 19B.

GENE EXPRESSION ANALYSIS: Expression of inflammatory and bone remodeling genes were determined by RT/PCR analysis. To perform the quantitative PCR, total RNA was isolated from hind paws using RNA STAT-60 (Tel-Test, Friendswood, Tex., USA). Total RNA (5  $\mu$ g) was subjected to treatment with DNase (Roche). DNase-treated total RNA was reverse-transcribed using Superscript II (Gibco/BRL). Primers were designed using Primer Express (PE Biosystems), or obtained commercially from Applied Biosystems.

Real-time quantitative PCR on 10 ng of cDNA from each sample was performed using one of two methods. In the first method, two gene-specific unlabelled primers were utilized at 400 nM in a Perkin Elmer SYBR green real-time quantitative PCR assay utilizing an ABI 5700 Instrument. In the second method, two unlabelled primers at 900 nM each were used with 250 nM of FAM-labelled probe (Applied Biosystems) in a TAQMAN™ real-time quantitative PCR reaction on an ABI 7700 sequence detection system. The absence of genomic

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DNA contamination was confirmed using primers that recognize genomic regions of the CD4 promoter-samples with detectable DNA contamination by real-time PCR were excluded from the study. Ubiquitin levels were measured in a separate reaction and used to normalize the data by the  $\Delta\text{-}\Delta\text{Ct}$  method, using the mean cycle threshold (Ct) value for ubiquitin and the genes of interest for each sample; the equation  $1.8^e (\text{Ct ubiquitin}-\text{Ct gene of interest})\times 10^4$  was used to obtain the normalized values. The results are shown in Table 8. Data shown is fold-increase of inflammatory/bone remodeling gene expression over that of gene expression in hind paws of naïve control mice.

TABLE 8

Fold induction over naïve mice						
Gene	Isotype Control 78ABY	Asialyated Humira 36ADV	$\alpha$ 2,3 Humira 19ADX	Gamma Guard 84ADU	TNFR-Ig 82ABW	$\alpha$ 2,6-high Humira 37ADV
IL-1b	47.09	25.75	58.94	1.58	1.76	4.55
IL-6	26.18	3.64	10.71	0.37	0.80	0.59
RankL	186.32	138.96	347.65	6.25	20.36	21.26
MDL-1	4.65	3.79	5.71	1.23	0.97	1.93
Cd11b	9.43	13.20	23.11	1.77	3.17	5.25
Dap12	4.45	3.54	6.01	1.81	1.59	2.26
TIMP-1	15.98	6.80	18.43	1.40	1.21	1.38
PU.1	6.91	9.18	15.50	1.00	1.48	3.17
Trap - Acp5	13.88	15.44	35.34	1.19	2.14	5.72
MCP1	14.96	5.66	11.62	1.46	1.44	1.59
Cxcl1 - Groa	23.04	4.92	18.10	0.39	1.34	1.41
Cxcl2 - Grob	30.83	6.30	16.06	2.93	1.15	0.99
Atp6v0d2	16.62	9.81	21.87	1.60	1.81	2.68
Mmp9	15.00	12.33	26.43	1.69	2.26	3.24
Fcgr1 - Cd64	5.84	4.48	8.68	1.87	1.59	2.36
Fcgr2b - Cd32a	3.63	2.81	4.34	1.48	1.59	1.60
Fcgr3	4.07	6.64	7.73	1.33	1.81	4.86
Fcgr4	8.91	7.79	14.10	2.93	2.12	4.15
Ctsk	13.13	15.04	30.55	1.25	2.20	4.17
Itgb2	6.46	7.66	17.07	1.16	1.68	3.24
MIP1a	5.12	3.15	5.19	1.39	1.08	1.47

## EXAMPLE 22

Fc $\gamma$ R Binding Assays of Anti-TNF Antibodies and Muteins

Fc $\gamma$  receptor binding assays were carried out as described in Example 1, using the anti-TNF antibodies described in Example 18. The results are shown in FIG. 20 and Tables 9-10.

TABLE 9

Reduction in Binding to Fc $\gamma$ receptor compared to wildtype (parent) antibody produced in GS 5.0					
Sample	Fc $\gamma$ RI	Fc $\gamma$ RIIa	Fc $\gamma$ RIIb/c	Fc $\gamma$ IIIa LF	Fc $\gamma$ IIIa LV
Anti-TNF DM (produced in GS5.0)	↓ 4-10	↓ 60-100	non-binding	↓ 11-12	↓ 1.5-1.7
Anti-TNF DM (produced in GS6.0)	↓ 2-3	↓ 100	non-binding	↓ 10-12	↓ 1.5-2.0

↓ indicates decreased affinity fold  
↑ indicates increased affinity fold

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TABLE 10

Reduction in Binding to Fc $\gamma$ receptor compared to commercial HUMIRA					
Sample	Fc $\gamma$ RI	Fc $\gamma$ RIIa	Fc $\gamma$ RIIb/c	Fc $\gamma$ IIIa LF	Fc $\gamma$ IIIa LV
Anti-TNF DM (produced in GS5.0)	↓ 4-11	non-binding	↓ 20	no difference	no difference
Anti-TNF DM (produced in GS6.0)	↓ 2-4	non-binding	↓ 20	no difference	no difference
Anti-TNF wildtype (produced in GS5.0)	no difference	↑ 30	↑ 2	↑ 13-15	↑ 1.8-2.0

↓ indicates decreased affinity fold  
↑ indicates increased affinity fold

## EXAMPLE 23

## Construction of Additional Anti-HER2 Fc Double Muteins (at Positions 243/264) and Their N-Glycan Composition

Additional Fc double muteins of the Her2 IgG1 antibody described in Example 2 were constructed using a Stratagene QuikChange® Site-Directed Mutagenesis Kit Catalog #200518 (30 reactions) following the protocol they provided to process saturation mutagenesis with degenerate primers. The signal sequence of an alpha-mating factor predomain was fused in frame to the 5' end of the light chain and heavy chain. The resulting heavy and light chains with the fused signal sequence of IgG1 and its muteins were cloned under *Pichia pastoris* AOX1 promoter and in front of *S. cerevisiae* Cyc terminator, respectively. The expression cassette of the completed heavy and light chains was put together into the final expression vector.

The vectors were expressed in glycoengineered *Pichia* GFI6.0 hosts cells YGLY3582 and YGLY22812.

TABLE 11

Strain	Description
YGLY4563	GFI6.0 host YGLY3582 strain making anti-Her2 F243A/V264A double mutein
YGLY23294	GFI6.0 host YGLY22812 strain making anti-Her2 F243Y/V264G double mutein
YGLY23280	GFI6.0 host YGLY22812 strain making anti-Her2 F243T/V264G double mutein
YGLY23301	GFI6.0 host YGLY22812 strain making anti-Her2 F243L/V264A double mutein
YGLY25259	GFI6.0 host YGLY22812 strain making anti-Her2 F243L/V264N double mutein
YGLY23305	GFI6.0 host YGLY22812 strain making anti-Her2 F243V/V264G double mutein

The genotype of the GFI6.0 YGLY3582 strain is described at Example 4 (same as YGLY4563).

The genotype of the engineered *Pichia pastoris* GFI 6.0 YGLY22812 strain used for the expression of the anti-TNF $\alpha$  Fc DM mutein, is as follows: *ura5 $\Delta$ ::ScSUC2 och1 $\Delta$ ::lacZ bmt2 $\Delta$ ::lacZ/KIMNN2-2 mnn4L1 $\Delta$ ::lacZ/MmSLC35A3 pno1 $\Delta$  mnn4 $\Delta$ ::lacZ ADE1::lacZ/NA10/MmSLC35A3/FB8his1 $\Delta$ ::lacZ/ScGAL10/XB33/DmUGT arg1 $\Delta$ ::HIS1/KD53/TC54bmt4 $\Delta$ ::lacZ bmt1 $\Delta$ ::lacZ bmt3 $\Delta$ ::lacZ TRP2: ARG1/MmCST/HsGNE/HsCSS/HsSPS/MmST6-33ste13 $\Delta$ ::lacZ/TrMDS1 dap2 $\Delta$ ::NatR TRP5: HygRMmCST/HsGNE/HsCSS/HsSPS/MmST6-33 Vps10-1 $\Delta$ :: AOX1p\_LmSTT3d. YGLY23294, YGLY23280, YGLY23301, YGLY25259 and YGLY23305 have the same genotype except they express different mutein listed in Table 11.*

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The strains were cultivated in 500 ml shake flasks with 300 ml of 2% BMGY media and shaken at 24°C. for 3 days.

Protocol for Induction of Shake Flasks: Collect the total volume of each culture (300 ml) into falcon tubes and spin at 2500 rpm for 5 minutes. Pour away supernatant and resuspend cell pellets in a final volume of 150 ml 2% BMMY and 360 ul PMTi4 (stock concentration 0.65 mg/ml). Transfer to a fresh 500 ml shake flask and shake at 24°C. for 2 days. Spin down the induced cultures and collect the supernatant into fresh falcon tubes.

The secreted antibodies were purified by protein A column using GE Healthcare, STREAMLINE rProtein A (catalog no. 17-1281-01) and BioRad poly-prep chromatography columns (10 ml) (catalog no. 731-1550). The following buffers were used:

Wash buffer #1: 20 mM Tris pH 7.0, 1M NaCl

Wash buffer/2: 20 mM Tris pH 7.0

Neutralization buffer: 1M Tris pH 8.0-pH 9.0

Elution buffer: 100 mM or 50 mM Sodium Citrate pH 3.0

Cleaning solution: 6M Urea in water.

The purification protocol is as follows:

Add 500 ul of STREAMLINE rProtein A beads to each BioRad column. The beads should be in 20% ethanol. The composition of the bead slurry should be 50% beads, 50% liquid.

Once the protein A beads are in the column they should be washed with 5 mls of Wash buffer #2 (discard the flow through)

Add 10 mls of supernatant to the BioRad column. During this step the antibodies will bind to the protein A beads. (discard the flow through)

Wash away undesired excess proteins by adding 5 mls of Wash buffer #1 to the column. (discard the flow through)

Wash the column again by adding 5 mls of Wash buffer #2 (discard the flow through)

Add 1 ml of Neutralization buffer to the 15 ml protein collection tube.

Place the BioRad column into the 15 ml collection tube.

Add 3 mls of Elution buffer to the BioRad column. This will remove the desired antibodies from the protein A beads.

Collect the eluted protein in the 15 ml protein collection tube.

Determine the concentration of the eluted protein by Bradford assay (use 10 ul of protein for Bradford assay).

To quantify the relative amount of each glycoform, the N-glycosidase F released glycans were labeled with 2-aminobenzidine (2-AB) and analyzed by HPLC as described in Choi et al., *Proc. Natl. Acad. Sci. USA* 100: 5022-5027 (2003) and Hamilton et al., *Science* 313: 1441-1443 (2006). Table 12 shows glycan profile of the produced antibodies (NQP=no quantification possible).

TABLE 12

	M5	G2	M7	M8	A1	A1H	A2	HM
YGLY4563	2.4	3.2	2.8	2.5	7.9	9.6	67.3	4.3
YGLY23294	16.9	NQP	—	—	23.4	—	59.7	—
YGLY23280	20.6	NQP	—	—	16.3	—	63.1	—
YGLY23301	15.5	—	—	—	21.9	—	59.4	—
YGLY25259	27.7	NQP	—	—	23.9	—	48.3	—
YGLY23305	29.6	NQP	—	—	18.1	—	52.4	—

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The binding of the above mutants to various Fcγ receptors was determined using the method described in Example 11. The results are shown in FIG. 21 and Tables 13 and 14.

TABLE 13

Sample	Binding Affinity as compared to the F243A/V264A double mutein		
	FcγRIIb/c	FcγIIIa LF	FcγIIIa LV
YGLY23301	↑ 2	↑ 20	↑ 2.2
YGLY23305	↑ 12	↑ 15	↑ 3
YGLY23280	no change	↓ 4	↑ 3
YGLY23294	↑ 50	↑ >100	↑ 9
YGLY25259	no change	↑ >30	↑ 4

TABLE 14

Sample	Binding Affinity as compared to commercially available Herceptin		
	FcγRIIb/c	FcγIIIa LF	FcγIIIa LV
YGLY4563	Non-binding	↓ 8.4	no change
YGLY23301	↓ 3	↑ 2.4	↑ 2.2
YGLY23305	↑ 2	no change	no change
YGLY23280	↓ 3	↓ 25	↓ 6
YGLY23294	↑ >15	↑ 10	↑ 3.5
YGLY25259	↓ 2.5	↑ 2.5	no change

## EXAMPLE 23

## Anti-PCSK9 Parent Antibody and Fc Muteins

An anti-PCSK9 antibody having the heavy chain amino acid sequence of SEQ ID NO:13 and the light chain amino acid sequence of SEQ ID NO:14 was also constructed, and mutations F243A and V264A of the heavy chain were introduced as described in Example 2. The heavy chain amino acid sequence of the anti-PCSK9 double mutein is shown in SEQ ID NO:15. The wild-type and double mutein forms of the antibody were expressed in Glycoengineered *Pichia* GFI5.0 and GFI6.0, and purified according to the procedures described in Examples 3-7, and their ability to bind the various Fcγ receptors was determined using the procedures described in Example 11. The anti-PCSK9 double mutein antibody (produced in GFI6.0, comprising the heavy chain amino acid sequence of SEQ ID NO:15 and the light chain amino acid sequence of SEQ ID NO:14), when compared to the parent antibody (produced in GFI5.0) had approximately a 4 fold reduction in binding to FcγRI, a 8-60 fold reduction in binding to FcγRIIIa LF, a 2-6 fold reduction in binding to FcγRIIIa LV, and no detectable binding to FcγRIIa or FcγRIIb/c.

## EXAMPLE 24

## Effect of the Anti-Her2 Antibody and its Fc Muteins in a Collagen-Antibody Induced Arthritis (AIA) Model

MODEL INDUCTION: AIA (Antibody induced arthritis) is induced with a commercial ArthroGen-CIA® arthritogenic monoclonal antibody (purchased from Chondrex) consisting of a cocktail of 5 monoclonal antibodies, clone A2-10 (IgG2a), F10-21 (IgG2a), D8-6 (IgG2a), D1-2G (IgG2b), and D2-112 (IgG2b), that recognize the conserved epitopes on various species of type II collagen.

ANIMALS: 10 week old B10.RIII male mice which are susceptible to arthritis induction without additional of co-stimulatory factors were used. These animals were purchased from Jackson Laboratory.

CLINICAL SCORING: Paw swelling was measured daily post-induction of arthritis. The severity of the disease was graded on a 0-3 scale per paw as follows: 0, normal; 1, swelling of one digit; 2, swelling of two or more digits; 3, swelling of the entire paw. The maximal clinical score per mouse is 12.

STUDY DESIGN: Arthritis was induced by passive transfer of 3 mg of anti-CII mAb pathogen cocktail IV on day 0.

All groups of mice were dosed on day minus 1. The Clinical Score was monitored for 7 days. Groups of Mice were treated subcutaneously with following reagents:

Group/ Reagent	Lot	Dose
A. Naïve	***	***
B. Isotype IgG1	78ABY	33 mpk
C. Asialyated Anti-Her2	38ADV	33 mpk
D. $\alpha$ 2,6 Sialyated Anti-Her2	37ADV	33 mpk
E. muTNFR-Ig	82ABW	33 mpk

All Groups n=5 except group E which has an n=4

An isotype IgG1 antibody was used as a control. The antibody bound to mouse anti-hexon and had the designation "27F11".

The sample identified as "Asialyated Anti-Her2" corresponds to the anti-Her2 antibody comprising the F243A/V264A mutations produced in GFI 5.0 strain YGLY19709. The YGLY19709 strain was derived from the anti-Her2 antibody producing strain YGLY13979, which was transformed with plasmid pGLY6301 for expressing LsSTT3d. Strain YGLY13979 and plasmid pGLY6301 are described in patent application: WO 2010/099186.

The sample identified as " $\alpha$ 2,6 Sialyated Anti-Her2" corresponds to the anti-Her2 antibody comprising the F243A/V264A mutations produced in GFI 6.0 strain YGLY4563 (see Example 4).

mTNFR-Ig is an immunoadhesin (anti-TNF receptor Ig-fusion protein) comprising the extracellular domain of mTNFR2 connected to mIgG1Fc starting at the hinge and spanning the CH2 and the CH3 regions, and comprising the amino acid sequence of SEQ ID NO:16 produced in CHO cells.

The results of these experiments are shown in FIG. 22.

## SEQUENCE LISTING

SEQ ID NO:	Description	Sequence
1	Heavy chain amino acid sequence of wt anti-HER antibody	EVQLVESGGGLVQPGGSLRLSCAASGFNIKDTYIHWVRQAPGKGLEWVARIYPTNGYTRYADSVKGRFTISADTSKNTAYLQMNLSRAEDTAVYYCSRWGGDGEYAMDYWGQGLTVIVSSASTKGPSVFLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSKVHFFPAVLQSSGLYSLSSVTVTPSSSLGTQTYICNVNHKPSNTKVDKKEPKSCDKTHTCPPCPAPELGGPSVFLFPPKPKDTLMI SRTP EVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTTPVLDSDGSFFLYSKLTVDKSRWQQGNVFCFSVMHEALHNHYTQKLSLSLSPGK
2	Light chain amino acid sequence of anti-HER antibody	DIQMTQSPSSLSASVGRVITITCRASQDVTAVAWYQQKPKGKAPKLLIYSASFVLYSGVPSRFSGS RSGTDFLTITSSLPEDFATYYCQQHYTTPPTFGQGTKVEIKRTVAAPSVFIFPPSDEQLKSGTASVVCLLNFPYPREAKVQWKVDNALQSGNSQESVTEQDSKDYSLSTLTLSKADYEKHKVYACEVTHQGLSPVTKSFNRGEC
3	Primer	GGTCCTTCCGTTTTTTTTGGCCCCACCAAAGCCAAAGGACACTTTG
4	Primer	GTGTCCTTTGGCTTTGGTGGGGCCAAAAAACGGAAGGACCACC
5	Primer	gttacatgtgtgtgacggtttctcagag
6	Primer	GGTCCTCGTGAGAAACGTCAGCAACAACACATG
7	Alpha-mating factor DNA sequence	GAATTCGAAACGATGAGATTTCTCAATTTTACTGCTGTTTTATTTCGAGCATCTCCGCATTAGCT
8	Alpha-mating factor amino acid sequence	MRFPSIFTAVLFAASSALA
9	Heavy chain amino acid sequence of double mutein anti-HER antibody	EVQLVESGGGLVQPGGSLRLSCAASGFNIKDTYIHWVRQAPGKGLEWVARIYPTNGYTRYADSVKGRFTISADTSKNTAYLQMNLSRAEDTAVYYCSRWGGDGEYAMDYWGQGLTVIVSSASTKGPSVFLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSKVHFFPAVLQSSGLYSLSSVTVTPSSSLGTQTYICNVNHKPSNTKVDKKEPKSCDKTHTCPPCPAPELGGPSVFLFPPKPKDTLMI SRTP EVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTTPVLDSDGSFFLYSKLTVDKSRWQQGNVFCFSVMHEALHNHYTQKLSLSLSPGK

-continued

SEQUENCE LISTING		
SEQ ID NO:	Description	Sequence
10	heavy chain amino acid sequence of wildtype anti-TNF alpha antibody	E V Q L V E S G G G L V Q P G R S L R L S C A A S G F T F D D Y A M H W V R Q A P G K G L E W V S A I T W N S G H I D Y A D S V E G R F T I S R D N A K N S L Y L Q M N S L R A E D T A V Y Y C A K V S Y L S T A S S L D Y W G Q G T L V T V S S A S T K G P S V F P L A P S S K S T S G G T A A L G C L V K D Y F P E P V T V S W N S G A L T S G V H T F P A V L Q S S G L Y S L S S V V T V P S S S L G T Q T Y I C N V N H K P S N T K V D K K V E P K S C D K T H T C P P C P A P E L L G G R S V F L F P P K P K D T L M I S R T P E V T C V V V D V S H E D P E V K F N N Y V D G V E V H N A K T K P R E E Q Y N S T Y R V V S V L T V L H Q D W L N G K E Y K C K V S N K A L P A P I E K T I S K A K G Q P R E P Q V Y T L P P S R D E L T K N Q V S L T C L V K G F Y P S D I A V E W E S N G Q P E N N Y X T T P P V L D S D G S F F L Y S K L T V D K S R W Q Q G N V F S C S V M R E A L R N H Y T Q K S L S L S P G
11	Light chain amino acid sequence of anti-TNF alpha antibody	D I Q M T Q S P S S L S A S V G D R V T I T C R A S Q G I R N Y L A W Y Q Q K P G K A P K L L I Y A A S T L Q S G V P S R F S G S G S G T D F T L T I S S L Q P E D V A T Y Y C Q R Y N R A P Y T E G Q G T K V E I K R T V A A P S V F I F P P S D E Q L K S G T A S V V C L L N N F Y P R E A K V Q W K V D N A L Q S G N S Q E S V T E Q D S K D S T Y S L S S T L T L S K A D Y E K H K V Y A C E V T R Q G L S S P V T K S F N R G E C
12	Heavy chain amino acid sequence of double mteuin anti-TNF alpha antibody	E V Q L V E S G G G L V Q P G R S L R L S C A A S G F T F D D Y A M H W V R Q A P G K G L E W V S A I T W N S G H I D Y A D S V E G R F T I S R D N A K N S L Y L Q M N S L R A E D T A V Y Y C A K V S Y L S T A S S L D Y W G Q G T L V T V S S A S T K G P S V F P L A P S S K S T S G G T A A L G C L V K D Y F P E P V T V S W N S G A L T S G V R T F P A V L Q S S G L Y S L S S V V T V P S S S L G T Q T Y I C N V N H K P S N T K V D K K V E P K S C D K T H T C P P C P A P E L L G G P S V F L A P P K P K D T L M I S R T P E V T C V V A D V S H E D P E V K F N W Y V D G V E V H N A K T K P R E E Q Y N S T Y R V V S V L T V L H Q D W L N G K E Y K C K V S N K A L P A P I E K T I S K A K G Q P R E P Q V Y T L P P S R D E L T K N Q V S L T C L V K G F Y P S D I A V E W E S N G Q P E N N Y K T T P P V L D S D G S F F L Y S K L T V D K S R W Q Q G N V F S C S V M H E A L H N H Y T Q K S L S L S P G
13	Heavy chain amino acid sequence of wt anti-PCSK9 antibody	QVQLVQSGAEVKKPGASVKVSCKVSGYFTDYYMNWVRQAPGQGLEWIGDINPNNGGAIYNQKFK GRATLTVDKSTSTAYMELRSLRSDDTAVYYCTSGII TEIAEDFWGQGLVTV S S A S T K G P S V F P L A P S S K S T S G G T A A L G C L V K D Y F P E P V T V S W N S G A L T S G V H T F P A V L Q S S G L Y S L S S V V T V P S S S L G T Q T Y I C N V N H K P S N T K V D K K V E P K S C D K T H T C P P C P A P E L L G G P S V F L F P P K P K D T L M I S R T P E V T C V V V D V S H E D P E V K F N W Y V D G V E V H N A K T K P R F E Q Y N S T Y R V V S V L T V L H Q D W L N G K E Y K C K V S N K A L P A P I E K T I S K A K G Q P R E P Q V Y T L P P S R D E L T K N Q V S L T C L V K G F Y P S D I A V E W E S N G Q P E N N Y K T T P P V L D S D G S F F L Y S K L T V D K S E W Q Q G N V F S C S V M H E A L H N H Y T Q K S L S L S P G

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SEQUENCE LISTING		
SEQ ID NO:	Description	Sequence
14	Light chain amino acid sequence of wt anti-PCSK9 antibody	DIQMTQSPSSLSASVGDRVTITCKASQNVGTVVWVYQQKPGKAPKALIHSAASYRYSYSGVPSRFSGS GSGTDFTLTISSLPEDFATYYCQQYKTYPYTFGQGTKVEIKRTVAAPSVFIFPPSDEQLKSGTA SVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSSTYSLSSTLTLSKADYEKHKVYACE VTHQGLSSPVTKSFNRGEC
15	Heavy chain amino acid sequence of double mutein anti-PCSK9 antibody	QVQLVQSGAEVKKPGASVKVSCKVSGYFTDYYMNWVRQAPGGLEWIGDINPNNGGAIYNQKFK GRATLTVDKSTSTAYMELRSLRSDDTAVYYCTSGIITEIAEDFWGQGLVTV S S A S T K G P S V F P L A P S S K S T S G G T A A L G C L V K D Y F P E P V T V S W N S G A L T S G V H T F P A V L Q S S G L Y S L S S V V T V P S S S L G T Q T Y I C N V N H K P S N T K V D K K V E P K S C D K T H T C P P C P A P E L L G G P S V F L A P P K P K D T L M I S R T P E V T C V V A D V S H E D P E V K F N N Y V D G V E V H N A K T K P R E E Q Y N S T Y R V V S V L T V L H Q D W L N G K E Y K Q K V S N K A L P A P I E K T I S K A K G Q P R E P Q V Y T L P P S R D E L T K N Q V S L T C L V K G F Y P S D I A V F W E S N G Q P E N N Y K T T P P V L D S D G S F F L Y S K L T V D K S R W Q Q G N V F S C S V M H E A L H N H Y T Q K S L S L S P G
16	anti-TNF Ig-fusion protein	Vvltpykpepgyecqisqeydrkaqmccakcpggyvkhfcnktsdtvcadceasmytqwnqf rtclscsssttdqveiractkqqrvcaceagrycalkthsgscrqcmrlskcpgfgvassra pngnvlckacapgtfsdtstsdvcrphricsilaipgnastdavcapesptlsaiprtlyvsqp eptrsqpidgepgpsqtpsiltslgstpieqstkggsvprdcgckpcictvpevssvfifppk pkdvltitltpkvtcvvdiskddpevqfswfvddvevhtaqtkpreeqfnstfrsyselpimhq dwingkefkcrvnsaafpapiektisktkgrpkapqvtytipppkeqmakdkvsltcmidffped itvewqwnqpaenykntqpimtdtgsyfvysklvqksnweagntftcslheglhnhhteksl shspgk
17	Extracellular domain of FcγRIIb/c and histidine tag	M R F P S I F T A V L F A A S S A L A A P V N T T T E D E T A Q I P A F A V I G Y S D L E G D F D V A V L P F S N S T N N G L L F I N T T I A S I A A K E E G V S L E K R A P P K A V L K L E P Q W I N V L Q E D S V T L T C R G T H S P E S D S I Q W F H N G N L I P T H T Q P S Y R F K A N N N D S G E Y T C Q T G Q T S L S D P V H L T V L S E W L V L Q T P H L E F Q E G E T I V L R C H S W E D K P L V K V T F F Q N G K S K K F S R S D P N F S T P Q A N H S R S G D Y H C T G N I G Y T L Y S S E P V T I T V Q A P G G G H H H H H H H H H
18	Fc region	T C P P C P A P E L L G G P S V F L F P P K P K D T L M I S R T P E V T C V V V D V S H E D P E V K F N W Y V D G V E V H N A K T K P R E E Q Y N S T Y R V V S V L T V L H Q D W L N G K E Y K C K V S N K A L P A P I E K T I S K A K G Q P R E P Q V Y T L P P S R D E L T K N Q V S L T C L V K G F Y P S D I A V E W E S H G Q P E N N Y K T T P P V L D S D G S F F L Y S K L T V D K S R W Q Q G N V F S C S V M H E A L H N H Y T Q K S L S L S P G
19	Fc region	E P K S C D K T H T C P P C P A P E L L G G P S V F L F P P K P K D T L M I S R T P E V T C V V V D V S H E D P E V K F N W Y V D G V E V H N A K T K P R E E Q Y N S T Y R V V S V L T V L H Q D W L N G K E Y K C K V S N K A L P A P I E K T I S K A K G Q P R E P Q V Y T L P P S R D E L T

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## SEQUENCE LISTING

SEQ ID NO: Description	Sequence
	K N Q V S L T C L V K G F Y P S D I
	A V E W E S N G Q P E N N Y K T T P
	P V L D S D G S F F L Y S K L T V D
	K S R W Q Q G N V F S C S V M H E A
	L H N H Y T Q K S L S L S P G

While the present invention is described herein with reference to illustrated embodiments, it should be understood that the invention is not limited hereto. Those having ordinary skill in the art and access to the teachings herein will recognize additional modifications and embodiments within the scope thereof.

## SEQUENCE LISTING

&lt;160&gt; NUMBER OF SEQ ID NOS: 19

&lt;210&gt; SEQ ID NO 1

&lt;211&gt; LENGTH: 450

&lt;212&gt; TYPE: PRT

&lt;213&gt; ORGANISM: Artificial Sequence

&lt;220&gt; FEATURE:

&lt;223&gt; OTHER INFORMATION: antibody heavy chain

&lt;400&gt; SEQUENCE: 1

Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly Gly  
1 5 10 15

Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Phe Asn Ile Lys Asp Thr  
20 25 30

Tyr Ile His Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Val  
35 40 45

Ala Arg Ile Tyr Pro Thr Asn Gly Tyr Thr Arg Tyr Ala Asp Ser Val  
50 55 60

Lys Gly Arg Phe Thr Ile Ser Ala Asp Thr Ser Lys Asn Thr Ala Tyr  
65 70 75 80

Leu Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr Tyr Cys  
85 90 95

Ser Arg Trp Gly Gly Asp Gly Phe Tyr Ala Met Asp Tyr Trp Gly Gln  
100 105 110

Gly Thr Leu Val Thr Val Ser Ser Ala Ser Thr Lys Gly Pro Ser Val  
115 120 125

Phe Pro Leu Ala Pro Ser Ser Lys Ser Thr Ser Gly Gly Thr Ala Ala  
130 135 140

Leu Gly Cys Leu Val Lys Asp Tyr Phe Pro Glu Pro Val Thr Val Ser  
145 150 155 160

Trp Asn Ser Gly Ala Leu Thr Ser Gly Val His Thr Phe Pro Ala Val  
165 170 175

Leu Gln Ser Ser Gly Leu Tyr Ser Leu Ser Ser Val Val Thr Val Pro  
180 185 190

Ser Ser Ser Leu Gly Thr Gln Thr Tyr Ile Cys Asn Val Asn His Lys  
195 200 205

Pro Ser Asn Thr Lys Val Asp Lys Lys Val Glu Pro Lys Ser Cys Asp  
210 215 220

Lys Thr His Thr Cys Pro Pro Cys Pro Ala Pro Glu Leu Leu Gly Gly  
225 230 235 240

Pro Ser Val Phe Leu Phe Pro Pro Lys Pro Lys Asp Thr Leu Met Ile



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245				250				255							
Ser	Arg	Thr	Pro	Glu	Val	Thr	Cys	Val	Val	Val	Asp	Val	Ser	His	Glu
			260					265				270			
Asp	Pro	Glu	Val	Lys	Phe	Asn	Trp	Tyr	Val	Asp	Gly	Val	Glu	Val	His
		275					280					285			
Asn	Ala	Lys	Thr	Lys	Pro	Arg	Glu	Glu	Gln	Tyr	Asn	Ser	Thr	Tyr	Arg
		290				295					300				
Val	Val	Ser	Val	Leu	Thr	Val	Leu	His	Gln	Asp	Trp	Leu	Asn	Gly	Lys
305					310					315					320
Glu	Tyr	Lys	Cys	Lys	Val	Ser	Asn	Lys	Ala	Leu	Pro	Ala	Pro	Ile	Glu
				325					330					335	
Lys	Thr	Ile	Ser	Lys	Ala	Lys	Gly	Gln	Pro	Arg	Glu	Pro	Gln	Val	Tyr
			340					345					350		
Thr	Leu	Pro	Pro	Ser	Arg	Asp	Glu	Leu	Thr	Lys	Asn	Gln	Val	Ser	Leu
		355					360					365			
Thr	Cys	Leu	Val	Lys	Gly	Phe	Tyr	Pro	Ser	Asp	Ile	Ala	Val	Glu	Trp
		370				375					380				
Glu	Ser	Asn	Gly	Gln	Pro	Glu	Asn	Asn	Tyr	Lys	Thr	Thr	Pro	Pro	Val
385					390					395					400
Leu	Asp	Ser	Asp	Gly	Ser	Phe	Phe	Leu	Tyr	Ser	Lys	Leu	Thr	Val	Asp
				405					410					415	
Lys	Ser	Arg	Trp	Gln	Gln	Gly	Asn	Val	Phe	Ser	Cys	Ser	Val	Met	His
			420					425					430		
Glu	Ala	Leu	His	Asn	His	Tyr	Thr	Gln	Lys	Ser	Leu	Ser	Leu	Ser	Pro
		435					440					445			
Gly	Lys														
	450														

&lt;210&gt; SEQ ID NO 2

&lt;211&gt; LENGTH: 214

&lt;212&gt; TYPE: PRT

&lt;213&gt; ORGANISM: Artificial Sequence

&lt;220&gt; FEATURE:

&lt;223&gt; OTHER INFORMATION: antibody light chain

&lt;400&gt; SEQUENCE: 2

Asp	Ile	Gln	Met	Thr	Gln	Ser	Pro	Ser	Ser	Leu	Ser	Ala	Ser	Val	Gly
1				5					10					15	
Asp	Arg	Val	Thr	Ile	Thr	Cys	Arg	Ala	Ser	Gln	Asp	Val	Asn	Thr	Ala
			20					25					30		
Val	Ala	Trp	Tyr	Gln	Gln	Lys	Pro	Gly	Lys	Ala	Pro	Lys	Leu	Leu	Ile
		35					40					45			
Tyr	Ser	Ala	Ser	Phe	Leu	Tyr	Ser	Gly	Val	Pro	Ser	Arg	Phe	Ser	Gly
	50					55					60				
Ser	Arg	Ser	Gly	Thr	Asp	Phe	Thr	Leu	Thr	Ile	Ser	Ser	Leu	Gln	Pro
65					70					75					80
Glu	Asp	Phe	Ala	Thr	Tyr	Tyr	Cys	Gln	Gln	His	Tyr	Thr	Thr	Pro	Pro
				85					90					95	
Thr	Phe	Gly	Gln	Gly	Thr	Lys	Val	Glu	Ile	Lys	Arg	Thr	Val	Ala	Ala
			100					105					110		
Pro	Ser	Val	Phe	Ile	Phe	Pro	Pro	Ser	Asp	Glu	Gln	Leu	Lys	Ser	Gly
		115					120					125			
Thr	Ala	Ser	Val	Val	Cys	Leu	Leu	Asn	Asn	Phe	Tyr	Pro	Arg	Glu	Ala
	130					135					140				
Lys	Val	Gln	Trp	Lys	Val	Asp	Asn	Ala	Leu	Gln	Ser	Gly	Asn	Ser	Gln

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145	150	155	160
Glu Ser Val Thr	Glu Gln Asp Ser Lys Asp Ser Thr Tyr Ser Leu Ser		
	165	170	175
Ser Thr Leu Thr	Leu Ser Lys Ala Asp Tyr Glu Lys His Lys Val Tyr		
	180	185	190
Ala Cys Glu Val	Thr His Gln Gly Leu Ser Ser Pro Val Thr Lys Ser		
	195	200	205
Phe Asn Arg Gly	Glu Cys		
	210		
<210> SEQ ID NO 3			
<211> LENGTH: 45			
<212> TYPE: DNA			
<213> ORGANISM: Artificial Sequence			
<220> FEATURE:			
<223> OTHER INFORMATION: primer			
<400> SEQUENCE: 3			
ggtccttccg ttttttggc cccaccaaag ccaaggaca ctttg			45
<210> SEQ ID NO 4			
<211> LENGTH: 44			
<212> TYPE: DNA			
<213> ORGANISM: Artificial Sequence			
<220> FEATURE:			
<223> OTHER INFORMATION: primer			
<400> SEQUENCE: 4			
gtgtcctttg gctttggtg ggccaaaaa acggaaggac cacc			44
<210> SEQ ID NO 5			
<211> LENGTH: 33			
<212> TYPE: DNA			
<213> ORGANISM: Artificial Sequence			
<220> FEATURE:			
<223> OTHER INFORMATION: primer			
<400> SEQUENCE: 5			
gttacatgtg ttgttgctga cgtttctcac gag			33
<210> SEQ ID NO 6			
<211> LENGTH: 33			
<212> TYPE: DNA			
<213> ORGANISM: Artificial Sequence			
<220> FEATURE:			
<223> OTHER INFORMATION: primer			
<400> SEQUENCE: 6			
ggtcctcgtg agaaacgtca gcaacaacac atg			33
<210> SEQ ID NO 7			
<211> LENGTH: 69			
<212> TYPE: DNA			
<213> ORGANISM: Artificial Sequence			
<220> FEATURE:			
<223> OTHER INFORMATION: signal sequence			
<400> SEQUENCE: 7			
gaattcgaaa cgatgagatt tocttcaatt ttactgctg ttttattcgc agcatcctcc			60
gcattagct			69
<210> SEQ ID NO 8			
<211> LENGTH: 19			

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<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: signal sequence

<400> SEQUENCE: 8

Met Arg Phe Pro Ser Ile Phe Thr Ala Val Leu Phe Ala Ala Ser Ser
1           5           10           15

Ala Leu Ala

<210> SEQ ID NO 9
<211> LENGTH: 450
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: antibody heavy chain

<400> SEQUENCE: 9

Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly Gly
1           5           10           15

Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Phe Asn Ile Lys Asp Thr
20          25          30

Tyr Ile His Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Val
35          40          45

Ala Arg Ile Tyr Pro Thr Asn Gly Tyr Thr Arg Tyr Ala Asp Ser Val
50          55          60

Lys Gly Arg Phe Thr Ile Ser Ala Asp Thr Ser Lys Asn Thr Ala Tyr
65          70          75          80

Leu Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr Tyr Cys
85          90          95

Ser Arg Trp Gly Gly Asp Gly Phe Tyr Ala Met Asp Tyr Trp Gly Gln
100         105         110

Gly Thr Leu Val Thr Val Ser Ser Ala Ser Thr Lys Gly Pro Ser Val
115        120        125

Phe Pro Leu Ala Pro Ser Ser Lys Ser Thr Ser Gly Gly Thr Ala Ala
130        135        140

Leu Gly Cys Leu Val Lys Asp Tyr Phe Pro Glu Pro Val Thr Val Ser
145        150        155        160

Trp Asn Ser Gly Ala Leu Thr Ser Gly Val His Thr Phe Pro Ala Val
165        170        175

Leu Gln Ser Ser Gly Leu Tyr Ser Leu Ser Ser Val Val Thr Val Pro
180        185        190

Ser Ser Ser Leu Gly Thr Gln Thr Tyr Ile Cys Asn Val Asn His Lys
195        200        205

Pro Ser Asn Thr Lys Val Asp Lys Lys Val Glu Pro Lys Ser Cys Asp
210        215        220

Lys Thr His Thr Cys Pro Pro Cys Pro Ala Pro Glu Leu Leu Gly Gly
225        230        235        240

Pro Ser Val Phe Leu Ala Pro Pro Lys Pro Lys Asp Thr Leu Met Ile
245        250        255

Ser Arg Thr Pro Glu Val Thr Cys Val Val Ala Asp Val Ser His Glu
260        265        270

Asp Pro Glu Val Lys Phe Asn Trp Tyr Val Asp Gly Val Glu Val His
275        280        285

Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln Tyr Asn Ser Thr Tyr Arg
290        295        300

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Val Val Ser Val Leu Thr Val Leu His Gln Asp Trp Leu Asn Gly Lys  
 305 310 315 320  
 Glu Tyr Lys Cys Lys Val Ser Asn Lys Ala Leu Pro Ala Pro Ile Glu  
 325 330 335  
 Lys Thr Ile Ser Lys Ala Lys Gly Gln Pro Arg Glu Pro Gln Val Tyr  
 340 345 350  
 Thr Leu Pro Pro Ser Arg Asp Glu Leu Thr Lys Asn Gln Val Ser Leu  
 355 360 365  
 Thr Cys Leu Val Lys Gly Phe Tyr Pro Ser Asp Ile Ala Val Glu Trp  
 370 375 380  
 Glu Ser Asn Gly Gln Pro Glu Asn Asn Tyr Lys Thr Thr Pro Pro Val  
 385 390 395 400  
 Leu Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser Lys Leu Thr Val Asp  
 405 410 415  
 Lys Ser Arg Trp Gln Gln Gly Asn Val Phe Ser Cys Ser Val Met His  
 420 425 430  
 Glu Ala Leu His Asn His Tyr Thr Gln Lys Ser Leu Ser Leu Ser Pro  
 435 440 445  
 Gly Lys  
 450

<210> SEQ ID NO 10  
 <211> LENGTH: 450  
 <212> TYPE: PRT  
 <213> ORGANISM: Artificial Sequence  
 <220> FEATURE:  
 <223> OTHER INFORMATION: antibody heavy chain

<400> SEQUENCE: 10

Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly Arg  
 1 5 10 15  
 Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Phe Thr Phe Asp Asp Tyr  
 20 25 30  
 Ala Met His Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Val  
 35 40 45  
 Ser Ala Ile Thr Trp Asn Ser Gly His Ile Asp Tyr Ala Asp Ser Val  
 50 55 60  
 Glu Gly Arg Phe Thr Ile Ser Arg Asp Asn Ala Lys Asn Ser Leu Tyr  
 65 70 75 80  
 Leu Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr Tyr Cys  
 85 90 95  
 Ala Lys Val Ser Tyr Leu Ser Thr Ala Ser Ser Leu Asp Tyr Trp Gly  
 100 105 110  
 Gln Gly Thr Leu Val Thr Val Ser Ser Ala Ser Thr Lys Gly Pro Ser  
 115 120 125  
 Val Phe Pro Leu Ala Pro Ser Ser Lys Ser Thr Ser Gly Gly Thr Ala  
 130 135 140  
 Ala Leu Gly Cys Leu Val Lys Asp Tyr Phe Pro Glu Pro Val Thr Val  
 145 150 155 160  
 Ser Trp Asn Ser Gly Ala Leu Thr Ser Gly Val His Thr Phe Pro Ala  
 165 170 175  
 Val Leu Gln Ser Ser Gly Leu Tyr Ser Leu Ser Ser Val Val Thr Val  
 180 185 190  
 Pro Ser Ser Ser Leu Gly Thr Gln Thr Tyr Ile Cys Asn Val Asn His  
 195 200 205

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Lys Pro Ser Asn Thr Lys Val Asp Lys Lys Val Glu Pro Lys Ser Cys  
 210 215 220  
 Asp Lys Thr His Thr Cys Pro Pro Cys Pro Ala Pro Glu Leu Leu Gly  
 225 230 235 240  
 Gly Pro Ser Val Phe Leu Phe Pro Pro Lys Pro Lys Asp Thr Leu Met  
 245 250 255  
 Ile Ser Arg Thr Pro Glu Val Thr Cys Val Val Val Asp Val Ser His  
 260 265 270  
 Glu Asp Pro Glu Val Lys Phe Asn Trp Tyr Val Asp Gly Val Glu Val  
 275 280 285  
 His Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln Tyr Asn Ser Thr Tyr  
 290 295 300  
 Arg Val Val Ser Val Leu Thr Val Leu His Gln Asp Trp Leu Asn Gly  
 305 310 315 320  
 Lys Glu Tyr Lys Cys Lys Val Ser Asn Lys Ala Leu Pro Ala Pro Ile  
 325 330 335  
 Glu Lys Thr Ile Ser Lys Ala Lys Gly Gln Pro Arg Glu Pro Gln Val  
 340 345 350  
 Tyr Thr Leu Pro Pro Ser Arg Asp Glu Leu Thr Lys Asn Gln Val Ser  
 355 360 365  
 Leu Thr Cys Leu Val Lys Gly Phe Tyr Pro Ser Asp Ile Ala Val Glu  
 370 375 380  
 Trp Glu Ser Asn Gly Gln Pro Glu Asn Asn Tyr Lys Thr Thr Pro Pro  
 385 390 395 400  
 Val Leu Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser Lys Leu Thr Val  
 405 410 415  
 Asp Lys Ser Arg Trp Gln Gln Gly Asn Val Phe Ser Cys Ser Val Met  
 420 425 430  
 His Glu Ala Leu His Asn His Tyr Thr Gln Lys Ser Leu Ser Leu Ser  
 435 440 445  
 Pro Gly  
 450

<210> SEQ ID NO 11  
 <211> LENGTH: 214  
 <212> TYPE: PRT  
 <213> ORGANISM: Artificial Sequence  
 <220> FEATURE:  
 <223> OTHER INFORMATION: antibody light chain

<400> SEQUENCE: 11

Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val Gly  
 1 5 10 15  
 Asp Arg Val Thr Ile Thr Cys Arg Ala Ser Gln Gly Ile Arg Asn Tyr  
 20 25 30  
 Leu Ala Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys Leu Leu Ile  
 35 40 45  
 Tyr Ala Ala Ser Thr Leu Gln Ser Gly Val Pro Ser Arg Phe Ser Gly  
 50 55 60  
 Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu Gln Pro  
 65 70 75 80  
 Glu Asp Val Ala Thr Tyr Tyr Cys Gln Arg Tyr Asn Arg Ala Pro Tyr  
 85 90 95  
 Thr Phe Gly Gln Gly Thr Lys Val Glu Ile Lys Arg Thr Val Ala Ala  
 100 105 110

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Pro Ser Val Phe Ile Phe Pro Pro Ser Asp Glu Gln Leu Lys Ser Gly
   115                               120           125

Thr Ala Ser Val Val Cys Leu Leu Asn Asn Phe Tyr Pro Arg Glu Ala
   130                               135           140

Lys Val Gln Trp Lys Val Asp Asn Ala Leu Gln Ser Gly Asn Ser Gln
   145                               150           155           160

Glu Ser Val Thr Glu Gln Asp Ser Lys Asp Ser Thr Tyr Ser Leu Ser
   165                               170           175

Ser Thr Leu Thr Leu Ser Lys Ala Asp Tyr Glu Lys His Lys Val Tyr
   180                               185           190

Ala Cys Glu Val Thr His Gln Gly Leu Ser Ser Pro Val Thr Lys Ser
   195                               200           205

Phe Asn Arg Gly Glu Cys
   210

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<210> SEQ ID NO 12
<211> LENGTH: 450
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: antibody heavy chain

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<400> SEQUENCE: 12

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Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly Arg
 1                               5           10           15

Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Phe Thr Phe Asp Asp Tyr
   20                               25           30

Ala Met His Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Val
   35                               40           45

Ser Ala Ile Thr Trp Asn Ser Gly His Ile Asp Tyr Ala Asp Ser Val
   50                               55           60

Glu Gly Arg Phe Thr Ile Ser Arg Asp Asn Ala Lys Asn Ser Leu Tyr
   65                               70           75           80

Leu Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr Tyr Cys
   85                               90           95

Ala Lys Val Ser Tyr Leu Ser Thr Ala Ser Ser Leu Asp Tyr Trp Gly
  100                               105           110

Gln Gly Thr Leu Val Thr Val Ser Ser Ala Ser Thr Lys Gly Pro Ser
  115                               120           125

Val Phe Pro Leu Ala Pro Ser Ser Lys Ser Thr Ser Gly Gly Thr Ala
  130                               135           140

Ala Leu Gly Cys Leu Val Lys Asp Tyr Phe Pro Glu Pro Val Thr Val
  145                               150           155           160

Ser Trp Asn Ser Gly Ala Leu Thr Ser Gly Val His Thr Phe Pro Ala
  165                               170           175

Val Leu Gln Ser Ser Gly Leu Tyr Ser Leu Ser Ser Val Val Thr Val
  180                               185           190

Pro Ser Ser Ser Leu Gly Thr Gln Thr Tyr Ile Cys Asn Val Asn His
  195                               200           205

Lys Pro Ser Asn Thr Lys Val Asp Lys Lys Val Glu Pro Lys Ser Cys
  210                               215           220

Asp Lys Thr His Thr Cys Pro Pro Cys Pro Ala Pro Glu Leu Leu Gly
  225                               230           235           240

Gly Pro Ser Val Phe Leu Ala Pro Pro Lys Pro Lys Asp Thr Leu Met
  245                               250           255

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Ile Ser Arg Thr Pro Glu Val Thr Cys Val Val Ala Asp Val Ser His  
 260 265 270

Glu Asp Pro Glu Val Lys Phe Asn Trp Tyr Val Asp Gly Val Glu Val  
 275 280 285

His Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln Tyr Asn Ser Thr Tyr  
 290 295 300

Arg Val Val Ser Val Leu Thr Val Leu His Gln Asp Trp Leu Asn Gly  
 305 310 315 320

Lys Glu Tyr Lys Cys Lys Val Ser Asn Lys Ala Leu Pro Ala Pro Ile  
 325 330 335

Glu Lys Thr Ile Ser Lys Ala Lys Gly Gln Pro Arg Glu Pro Gln Val  
 340 345 350

Tyr Thr Leu Pro Pro Ser Arg Asp Glu Leu Thr Lys Asn Gln Val Ser  
 355 360 365

Leu Thr Cys Leu Val Lys Gly Phe Tyr Pro Ser Asp Ile Ala Val Glu  
 370 375 380

Trp Glu Ser Asn Gly Gln Pro Glu Asn Asn Tyr Lys Thr Thr Pro Pro  
 385 390 395 400

Val Leu Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser Lys Leu Thr Val  
 405 410 415

Asp Lys Ser Arg Trp Gln Gln Gly Asn Val Phe Ser Cys Ser Val Met  
 420 425 430

His Glu Ala Leu His Asn His Tyr Thr Gln Lys Ser Leu Ser Leu Ser  
 435 440 445

Pro Gly  
 450

<210> SEQ ID NO 13  
 <211> LENGTH: 448  
 <212> TYPE: PRT  
 <213> ORGANISM: Artificial Sequence  
 <220> FEATURE:  
 <223> OTHER INFORMATION: antibody heavy chain

<400> SEQUENCE: 13

Gln Val Gln Leu Val Gln Ser Gly Ala Glu Val Lys Lys Pro Gly Ala  
 1 5 10 15

Ser Val Lys Val Ser Cys Lys Val Ser Gly Tyr Thr Phe Thr Asp Tyr  
 20 25 30

Tyr Met Asn Trp Val Arg Gln Ala Pro Gly Gln Gly Leu Glu Trp Ile  
 35 40 45

Gly Asp Ile Asn Pro Asn Asn Gly Gly Ala Ile Tyr Asn Gln Lys Phe  
 50 55 60

Lys Gly Arg Ala Thr Leu Thr Val Asp Lys Ser Thr Ser Thr Ala Tyr  
 65 70 75 80

Met Glu Leu Arg Ser Leu Arg Ser Asp Asp Thr Ala Val Tyr Tyr Cys  
 85 90 95

Thr Ser Gly Ile Ile Thr Glu Ile Ala Glu Asp Phe Trp Gly Gln Gly  
 100 105 110

Thr Leu Val Thr Val Ser Ser Ala Ser Thr Lys Gly Pro Ser Val Phe  
 115 120 125

Pro Leu Ala Pro Ser Ser Lys Ser Thr Ser Gly Gly Thr Ala Ala Leu  
 130 135 140

Gly Cys Leu Val Lys Asp Tyr Phe Pro Glu Pro Val Thr Val Ser Trp  
 145 150 155 160

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Asn Ser Gly Ala Leu Thr Ser Gly Val His Thr Phe Pro Ala Val Leu
      165                               170                       175

Gln Ser Ser Gly Leu Tyr Ser Leu Ser Ser Val Val Thr Val Pro Ser
      180                               185                       190

Ser Ser Leu Gly Thr Gln Thr Tyr Ile Cys Asn Val Asn His Lys Pro
      195                               200                       205

Ser Asn Thr Lys Val Asp Lys Lys Val Glu Pro Lys Ser Cys Asp Lys
      210                               215                       220

Thr His Thr Cys Pro Pro Cys Pro Ala Pro Glu Leu Leu Gly Gly Pro
      225                               230                       235                       240

Ser Val Phe Leu Phe Pro Pro Lys Pro Lys Asp Thr Leu Met Ile Ser
      245                               250                       255

Arg Thr Pro Glu Val Thr Cys Val Val Val Asp Val Ser His Glu Asp
      260                               265                       270

Pro Glu Val Lys Phe Asn Trp Tyr Val Asp Gly Val Glu Val His Asn
      275                               280                       285

Ala Lys Thr Lys Pro Arg Glu Glu Gln Tyr Asn Ser Thr Tyr Arg Val
      290                               295                       300

Val Ser Val Leu Thr Val Leu His Gln Asp Trp Leu Asn Gly Lys Glu
      305                               310                       315                       320

Tyr Lys Cys Lys Val Ser Asn Lys Ala Leu Pro Ala Pro Ile Glu Lys
      325                               330                       335

Thr Ile Ser Lys Ala Lys Gly Gln Pro Arg Glu Pro Gln Val Tyr Thr
      340                               345                       350

Leu Pro Pro Ser Arg Asp Glu Leu Thr Lys Asn Gln Val Ser Leu Thr
      355                               360                       365

Cys Leu Val Lys Gly Phe Tyr Pro Ser Asp Ile Ala Val Glu Trp Glu
      370                               375                       380

Ser Asn Gly Gln Pro Glu Asn Asn Tyr Lys Thr Thr Pro Pro Val Leu
      385                               390                       395                       400

Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser Lys Leu Thr Val Asp Lys
      405                               410                       415

Ser Arg Trp Gln Gln Gly Asn Val Phe Ser Cys Ser Val Met His Glu
      420                               425                       430

Ala Leu His Asn His Tyr Thr Gln Lys Ser Leu Ser Leu Ser Pro Gly
      435                               440                       445

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<210> SEQ ID NO 14
<211> LENGTH: 214
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: antibody light chain

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<400> SEQUENCE: 14

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Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val Gly
1           5           10           15

Asp Arg Val Thr Ile Thr Cys Lys Ala Ser Gln Asn Val Gly Thr Asn
20          25          30

Val Val Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys Ala Leu Ile
35          40          45

His Ser Ala Ser Tyr Arg Tyr Ser Gly Val Pro Ser Arg Phe Ser Gly
50          55          60

Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu Gln Pro
65          70          75          80

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Glu Asp Phe Ala Thr Tyr Tyr Cys Gln Gln Tyr Lys Thr Tyr Pro Tyr  
85 90 95

Thr Phe Gly Gln Gly Thr Lys Val Glu Ile Lys Arg Thr Val Ala Ala  
100 105 110

Pro Ser Val Phe Ile Phe Pro Pro Ser Asp Glu Gln Leu Lys Ser Gly  
115 120 125

Thr Ala Ser Val Val Cys Leu Leu Asn Asn Phe Tyr Pro Arg Glu Ala  
130 135 140

Lys Val Gln Trp Lys Val Asp Asn Ala Leu Gln Ser Gly Asn Ser Gln  
145 150 155 160

Glu Ser Val Thr Glu Gln Asp Ser Lys Asp Ser Thr Tyr Ser Leu Ser  
165 170 175

Ser Thr Leu Thr Leu Ser Lys Ala Asp Tyr Glu Lys His Lys Val Tyr  
180 185 190

Ala Cys Glu Val Thr His Gln Gly Leu Ser Ser Pro Val Thr Lys Ser  
195 200 205

Phe Asn Arg Gly Glu Cys  
210

&lt;210&gt; SEQ ID NO 15

&lt;211&gt; LENGTH: 448

&lt;212&gt; TYPE: PRT

&lt;213&gt; ORGANISM: Artificial Sequence

&lt;220&gt; FEATURE:

&lt;223&gt; OTHER INFORMATION: antibody heavy chain

&lt;400&gt; SEQUENCE: 15

Gln Val Gln Leu Val Gln Ser Gly Ala Glu Val Lys Lys Pro Gly Ala  
1 5 10 15

Ser Val Lys Val Ser Cys Lys Val Ser Gly Tyr Thr Phe Thr Asp Tyr  
20 25 30

Tyr Met Asn Trp Val Arg Gln Ala Pro Gly Gln Gly Leu Glu Trp Ile  
35 40 45

Gly Asp Ile Asn Pro Asn Asn Gly Gly Ala Ile Tyr Asn Gln Lys Phe  
50 55 60

Lys Gly Arg Ala Thr Leu Thr Val Asp Lys Ser Thr Ser Thr Ala Tyr  
65 70 75 80

Met Glu Leu Arg Ser Leu Arg Ser Asp Asp Thr Ala Val Tyr Tyr Cys  
85 90 95

Thr Ser Gly Ile Ile Thr Glu Ile Ala Glu Asp Phe Trp Gly Gln Gly  
100 105 110

Thr Leu Val Thr Val Ser Ser Ala Ser Thr Lys Gly Pro Ser Val Phe  
115 120 125

Pro Leu Ala Pro Ser Ser Lys Ser Thr Ser Gly Gly Thr Ala Ala Leu  
130 135 140

Gly Cys Leu Val Lys Asp Tyr Phe Pro Glu Pro Val Thr Val Ser Trp  
145 150 155 160

Asn Ser Gly Ala Leu Thr Ser Gly Val His Thr Phe Pro Ala Val Leu  
165 170 175

Gln Ser Ser Gly Leu Tyr Ser Leu Ser Ser Val Val Thr Val Pro Ser  
180 185 190

Ser Ser Leu Gly Thr Gln Thr Tyr Ile Cys Asn Val Asn His Lys Pro  
195 200 205

Ser Asn Thr Lys Val Asp Lys Lys Val Glu Pro Lys Ser Cys Asp Lys  
210 215 220

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Thr His Thr Cys Pro Pro Cys Pro Ala Pro Glu Leu Leu Gly Gly Pro
225                230                235                240

Ser Val Phe Leu Ala Pro Pro Lys Pro Lys Asp Thr Leu Met Ile Ser
                245                250                255

Arg Thr Pro Glu Val Thr Cys Val Val Ala Asp Val Ser His Glu Asp
                260                265                270

Pro Glu Val Lys Phe Asn Trp Tyr Val Asp Gly Val Glu Val His Asn
                275                280                285

Ala Lys Thr Lys Pro Arg Glu Glu Gln Tyr Asn Ser Thr Tyr Arg Val
                290                295                300

Val Ser Val Leu Thr Val Leu His Gln Asp Trp Leu Asn Gly Lys Glu
305                310                315                320

Tyr Lys Cys Lys Val Ser Asn Lys Ala Leu Pro Ala Pro Ile Glu Lys
                325                330                335

Thr Ile Ser Lys Ala Lys Gly Gln Pro Arg Glu Pro Gln Val Tyr Thr
                340                345                350

Leu Pro Pro Ser Arg Asp Glu Leu Thr Lys Asn Gln Val Ser Leu Thr
                355                360                365

Cys Leu Val Lys Gly Phe Tyr Pro Ser Asp Ile Ala Val Glu Trp Glu
                370                375                380

Ser Asn Gly Gln Pro Glu Asn Asn Tyr Lys Thr Thr Pro Pro Val Leu
385                390                395                400

Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser Lys Leu Thr Val Asp Lys
                405                410                415

Ser Arg Trp Gln Gln Gly Asn Val Phe Ser Cys Ser Val Met His Glu
                420                425                430

Ala Leu His Asn His Tyr Thr Gln Lys Ser Leu Ser Leu Ser Pro Gly
                435                440                445

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<210> SEQ ID NO 16
<211> LENGTH: 461
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Fc fusion protein

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<400> SEQUENCE: 16

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Val Val Leu Thr Pro Tyr Lys Pro Glu Pro Gly Tyr Glu Cys Gln Ile
1                5                10                15

Ser Gln Glu Tyr Tyr Asp Arg Lys Ala Gln Met Cys Cys Ala Lys Cys
                20                25                30

Pro Pro Gly Gln Tyr Val Lys His Phe Cys Asn Lys Thr Ser Asp Thr
                35                40                45

Val Cys Ala Asp Cys Glu Ala Ser Met Tyr Thr Gln Val Trp Asn Gln
50                55                60

Phe Arg Thr Cys Leu Ser Cys Ser Ser Ser Cys Thr Thr Asp Gln Val
65                70                75                80

Glu Ile Arg Ala Cys Thr Lys Gln Gln Asn Arg Val Cys Ala Cys Glu
                85                90                95

Ala Gly Arg Tyr Cys Ala Leu Lys Thr His Ser Gly Ser Cys Arg Gln
                100                105                110

Cys Met Arg Leu Ser Lys Cys Gly Pro Gly Phe Gly Val Ala Ser Ser
                115                120                125

Arg Ala Pro Asn Gly Asn Val Leu Cys Lys Ala Cys Ala Pro Gly Thr
                130                135                140

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Asp Val Ala Val Leu Pro Phe Ser Asn Ser Thr Asn Asn Gly Leu Leu
 50                               55                               60

Phe Ile Asn Thr Thr Ile Ala Ser Ile Ala Ala Lys Glu Glu Gly Val
 65                               70                               75                               80

Ser Leu Glu Lys Arg Ala Pro Pro Lys Ala Val Leu Lys Leu Glu Pro
                               85                               90                               95

Gln Trp Ile Asn Val Leu Gln Glu Asp Ser Val Thr Leu Thr Cys Arg
                               100                              105                              110

Gly Thr His Ser Pro Glu Ser Asp Ser Ile Gln Trp Phe His Asn Gly
                               115                              120                              125

Asn Leu Ile Pro Thr His Thr Gln Pro Ser Tyr Arg Phe Lys Ala Asn
 130                               135                               140

Asn Asn Asp Ser Gly Glu Tyr Thr Cys Gln Thr Gly Gln Thr Ser Leu
 145                               150                               155                               160

Ser Asp Pro Val His Leu Thr Val Leu Ser Glu Trp Leu Val Leu Gln
                               165                               170                               175

Thr Pro His Leu Glu Phe Gln Glu Gly Glu Thr Ile Val Leu Arg Cys
                               180                              185                              190

His Ser Trp Lys Asp Lys Pro Leu Val Lys Val Thr Phe Phe Gln Asn
 195                               200                               205

Gly Lys Ser Lys Lys Phe Ser Arg Ser Asp Pro Asn Phe Ser Ile Pro
 210                               215                               220

Gln Ala Asn His Ser His Ser Gly Asp Tyr His Cys Thr Gly Asn Ile
 225                               230                               235                               240

Gly Tyr Thr Leu Tyr Ser Ser Lys Pro Val Thr Ile Thr Val Gln Ala
                               245                               250                               255

Pro Gly Gly Gly His His His His His His His His His His
 260                               265

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<210> SEQ ID NO 18
<211> LENGTH: 222
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Fc region

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<400> SEQUENCE: 18

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Thr Cys Pro Pro Cys Pro Ala Pro Glu Leu Leu Gly Gly Pro Ser Val
 1                               5                               10                               15

Phe Leu Phe Pro Pro Lys Pro Lys Asp Thr Leu Met Ile Ser Arg Thr
                               20                               25                               30

Pro Glu Val Thr Cys Val Val Val Asp Val Ser His Glu Asp Pro Glu
 35                               40                               45

Val Lys Phe Asn Trp Tyr Val Asp Gly Val Glu Val His Asn Ala Lys
 50                               55                               60

Thr Lys Pro Arg Glu Glu Gln Tyr Asn Ser Thr Tyr Arg Val Val Ser
 65                               70                               75                               80

Val Leu Thr Val Leu His Gln Asp Trp Leu Asn Gly Lys Glu Tyr Lys
                               85                               90                               95

Cys Lys Val Ser Asn Lys Ala Leu Pro Ala Pro Ile Glu Lys Thr Ile
                               100                              105                              110

Ser Lys Ala Lys Gly Gln Pro Arg Glu Pro Gln Val Tyr Thr Leu Pro
 115                               120                               125

Pro Ser Arg Asp Glu Leu Thr Lys Asn Gln Val Ser Leu Thr Cys Leu
 130                               135                               140

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Val Lys Gly Phe Tyr Pro Ser Asp Ile Ala Val Glu Trp Glu Ser Asn
145                               150                               155                               160

Gly Gln Pro Glu Asn Asn Tyr Lys Thr Thr Pro Pro Val Leu Asp Ser
                               165                               170                               175

Asp Gly Ser Phe Phe Leu Tyr Ser Lys Leu Thr Val Asp Lys Ser Arg
                               180                               185                               190

Trp Gln Gln Gly Asn Val Phe Ser Cys Ser Val Met His Glu Ala Leu
                               195                               200                               205

His Asn His Tyr Thr Gln Lys Ser Leu Ser Leu Ser Pro Gly
                               210                               215                               220

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<210> SEQ ID NO 19
<211> LENGTH: 231
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Fc region

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<400> SEQUENCE: 19

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Glu Pro Lys Ser Cys Asp Lys Thr His Thr Cys Pro Pro Cys Pro Ala
1                               5                               10                               15

Pro Glu Leu Leu Gly Gly Pro Ser Val Phe Leu Phe Pro Pro Lys Pro
                               20                               25                               30

Lys Asp Thr Leu Met Ile Ser Arg Thr Pro Glu Val Thr Cys Val Val
                               35                               40                               45

Val Asp Val Ser His Glu Asp Pro Glu Val Lys Phe Asn Trp Tyr Val
                               50                               55                               60

Asp Gly Val Glu Val His Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln
65                               70                               75                               80

Tyr Asn Ser Thr Tyr Arg Val Val Ser Val Leu Thr Val Leu His Gln
                               85                               90                               95

Asp Trp Leu Asn Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn Lys Ala
                               100                              105                              110

Leu Pro Ala Pro Ile Glu Lys Thr Ile Ser Lys Ala Lys Gly Gln Pro
                               115                              120                              125

Arg Glu Pro Gln Val Tyr Thr Leu Pro Pro Ser Arg Asp Glu Leu Thr
                               130                              135                              140

Lys Asn Gln Val Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr Pro Ser
145                              150                              155                              160

Asp Ile Ala Val Glu Trp Glu Ser Asn Gly Gln Pro Glu Asn Asn Tyr
                               165                              170                              175

Lys Thr Thr Pro Pro Val Leu Asp Ser Asp Gly Ser Phe Phe Leu Tyr
                               180                              185                              190

Ser Lys Leu Thr Val Asp Lys Ser Arg Trp Gln Gln Gly Asn Val Phe
                               195                              200                              205

Ser Cys Ser Val Met His Glu Ala Leu His Asn His Tyr Thr Gln Lys
                               210                              215                              220

Ser Leu Ser Leu Ser Pro Gly
225                              230

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What is claimed:

1. An Fc-containing polypeptide comprising the amino acid sequence of SEQ ID NO: 18, wherein said SEQ ID NO:18 comprises mutations at amino acid positions 243 and 264 of the Fc region, wherein the mutations are F243A and V264A, wherein the numbering is according to the EU index

<sup>60</sup> as in Kabat, and wherein the Fc-containing polypeptide is an antibody or antibody fragment comprising sialylated N-glycans.

<sup>65</sup> 2. The Fc-containing polypeptide of claim 1, wherein the sialic acid residues in the sialylated N-glycans are attached via  $\alpha$ -2,6 linkages.

3. The Fc-containing polypeptide of claim 1 or 2, wherein the Fc-containing polypeptide has one or more of the following properties when compared to a parent Fc-containing polypeptide:

- a) reduced effector function, 5
- b) increased anti-inflammatory properties,
- c) increased sialylation,
- d) increased bioavailability when administered parenterally, and
- e) reduced binding to FcγRI, FcγRIIa, FcγRIIb and 10 FcγRIIIa.

4. The Fc-containing polypeptide of claim 1, wherein at least 70 mole % of the N-glycans in the Fc-containing polypeptide have a structure selected from the group consisting of SA(1-4)Gal(1-4)GlcNAc((2-4)Man3GlcNAc2. 15

5. The Fc-containing polypeptide of claim 1, wherein at least 90 mole % of the N-glycans in the Fc-containing polypeptide have a structure selected from the group consisting of SA(1-4)Gal(1-4)GlcNAc((2-4)Man3GlcNAc2.

6. The Fc-containing polypeptide of claim 1, wherein at 20 least 66 mole % of the N-glycans in the Fc-containing polypeptide have a NANA2Gal2GlcNAc2Man3GlcNAc2 structure.

7. The Fc-containing polypeptide of claim 1, wherein at 25 least 90 mole % of the N-glycans in the Fc-containing polypeptide have a structure selected from the group consisting of SA(1-4)Gal(1-4)GlcNAc((2-4)Man3GlcNAc2.

8. The Fc-containing polypeptide of claim 1, wherein at 30 least 66 mole % of the N-glycans in the Fc-containing polypeptide have a NANA2Gal2GlcNAc2Man3GlcNAc2 structure.

\* \* \* \* \*

UNITED STATES PATENT AND TRADEMARK OFFICE  
**CERTIFICATE OF CORRECTION**

PATENT NO. : 9,187,552 B2  
APPLICATION NO. : 13/699213  
DATED : November 17, 2015  
INVENTOR(S) : Stadheim et al.

Page 1 of 1

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

On the Title Page

Item (75) Inventor is corrected to read:  
-- Terrance A. Stadheim, Lyme (NH);  
Dongxing Zha, Etna (NH) --.

Signed and Sealed this  
Tenth Day of October, 2017



Joseph Matal  
*Performing the Functions and Duties of the  
Under Secretary of Commerce for Intellectual Property and  
Director of the United States Patent and Trademark Office*